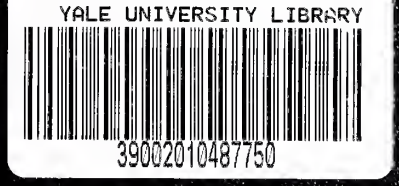

ABNORMALITIES IN HEPATOCYTE ENDOCYTOSIS IN CHRONIC PANCREATITIS

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1998

Abnormalities in Hepatocyte Endocytosis in Chronic Pancreatitis

A Thesis Submitted to the

Yale University School of Medicine

in Partial Fulfillment of the Requirements for the

Degree of Doctor of Medicine

by

Jaimie David Nathan

1998

ABNORMALITIES IN HEPATOCYTE ENDOCYTOSIS IN CHRONIC PANCREATITIS. Jaimie D. Nathan, Peter D. Zdankiewicz, Jinping Wang, Neal E. Seymour, John P. Geibel, Bhanu P. Jena, Dana K. Andersen. Departments of Surgery and Physiology, Yale University School of Medicine, New Haven, CT.

Chronic pancreatitis (CP) is associated with impaired glucose tolerance, reduced hepatic sensitivity to insulin, and a loss of insulin receptor (IR) availability on hepatocyte plasma membranes. The insulin-mediated reduction in the hepatocyte membrane-bound glucose transporter protein GLUT2 is impaired in CP as well. These abnormalities may be the result of altered intracellular vesicle trafficking. Hepatocyte fluid-phase endocytosis (FPE) was therefore assessed by *in vivo* uptake of FITC-Dextran (FITC-D) by confocal microscopy in livers from fed and fasting sham-operated (sham) rats and rats in which CP had been induced 2-3 months earlier by pancreatic duct oleic acid infusion. FITC-D uptake was greatly reduced in sham rats allowed access to chow *ad libitum*, compared to fasting rats ($p < 0.001$). In another experiment, 45 minutes after duodenal feeding in sham rats, FITC-D uptake remained low compared to fasting livers ($p < 0.05$). No significant inhibition of FITC-D uptake was seen in CP hepatocytes after either mode of feeding. To determine whether GLUT2 is actively internalized in hepatocytes and whether this receptor-mediated endocytosis (RME)-associated event is disordered in CP, livers were fractionated by sucrose density gradient ultracentrifugation to yield endosome (E)- and plasma membrane (PM)-enriched fractions, and GLUT2 content was quantified by Western blotting and scanning densitometry. Compared to fasting sham rats, the E:PM ratio of GLUT2 increased sharply in fed sham rats ($p < 0.05$). There was no change in the E:PM ratio of GLUT2 in CP livers following duodenal feeding. To test our findings using confocal microscopy, GLUT2 immunofluorescence was quantified by mean pixel intensity in an 8 x 16-pixel area of PM and a 16 x 16-pixel area of cytosol (CYT) in each of 30 random cells/field (400x) in each of 3 rats per group. Duodenal feeding increased the CYT:PM ratio of GLUT2, compared to fasting sham rats ($p < 0.0001$), while the CYT:PM ratio in CP remained unchanged. We conclude that following feeding, rat hepatocytes *in vivo* exhibit a reduction in FPE, and an increase in RME which induces a shift in GLUT2 from the plasma membrane to the endosomal pool. Both the feeding-induced reduction in FPE and internalization of GLUT2 are lost in hepatocytes from CP rats, suggesting that the more central abnormality may be a failure of hepatocytes to convert from FPE to RME. Disordered regulation of endocytosis and impaired GLUT2 internalization may play a role in the glucose intolerance associated with CP.

ACKNOWLEDGEMENTS

I am very grateful to Dana K. Andersen, M.D., for his insight, dedication, support and encouragement in the completion of this work. I thank Jinping Wang, M.D., for her tremendous expertise in the laboratory, and Peter D. Zdankiewicz, M.D., for his assistance, enthusiasm and friendship. I am grateful to Neal E. Seymour, M.D., and Bhanu P. Jena, Ph.D., for their guidance and suggestions, and to John P. Geibel, M.D., D.Sc., for his guidance and for the use of the confocal laser scanning microscopy equipment. I also thank William B. Stewart, Ph.D., for the use of his microtome equipment, and the Office of Student Research for the generous financial support. This work was supported by grant DK-39950 from the National Institutes of Health.

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INTRODUCTION

Pancreatogenic Diabetes

In 1788, Cawley provided the first report of diabetes mellitus resulting from inflammatory injury of the pancreas (1). One century later, Von Mering and Minkowski demonstrated that glucose intolerance, or even frank diabetes, develops following resection of the pancreas (2). The impaired glucose tolerance that accompanies pathologic destruction or surgical resection of the pancreas is referred to as pancreatogenic diabetes. Studies have shown that, clinically, pancreatogenic diabetes differs in several respects from the more common Type I (insulin-dependent) diabetes. Patients with diabetes secondary to pancreatic injury or resection are less likely to develop diabetic ketoacidosis and are more prone to hypoglycemic episodes following exogenous insulin administration (3-6). The result is rapid, sudden fluctuations in blood glucose levels and difficulty in maintaining glycemic control—so called "brittle" diabetes. In addition, the classic diabetic complications of microangiopathy, retinopathy and nephropathy are significantly less common than in Type I diabetics (3,7,8).

Chronic Pancreatitis

Chronic pancreatitis (CP) is frequently associated with pancreatogenic diabetes. Indeed, it has been known since Cawley's report in 1788 that an association exists between CP and diabetes mellitus. The discovery of insulin by Banting and Best was

inspired by a study describing the histologic changes that occur in the pancreas in CP (9). In 1946, Comfort et al. described the findings of hyperglycemia and glucosuria in patients with CP (10). However, few studies on diabetes secondary to CP were published before the 1980's when a Danish group led by Larsen began to investigate the disease systematically (11).

Chronic pancreatitis generally develops within the 3rd and 4th decades of life, however, the prevalence rate of CP has been difficult to determine for two major reasons. Variability in the level of alcohol consumption, the primary etiologic factor for the development of CP, has produced widely differing rates of CP throughout the world. In addition, the diagnostic criteria used for CP are not uniform from one setting to the next. Estimates from autopsy studies have suggested a mean prevalence of CP of approximately 13%, with a range from 0.2% to 66% (12). Similarly, differing diagnostic criteria and variable severity and duration of CP have made it difficult to determine the prevalence of diabetes secondary to CP. Studies have estimated, however, that approximately 70% of CP patients manifest impaired oral glucose tolerance, and 20% to 35% of pancreatitic patients develop overt diabetes mellitus and require insulin therapy (13-15). Diabetes may develop within one to 12 years following the diagnosis of CP, and on average, the onset of diabetes in pancreatitic patients occurs later in life than in patients with Type I diabetes (6,16).

It is well-known that genetic and autoimmune factors play a role in the pathogenesis of Type I diabetes (17). Although some studies have demonstrated islet cell antibodies in the sera of patients with CP, no association has been found between the presence of these antibodies and the development of diabetes (18-20). Furthermore,

neither CP nor the development of diabetes secondary to CP has been shown to be associated with specific HLA types, in contrast to Type I diabetes (21). Thus, no evidence exists to suggest that hereditary or immunological factors play a role in the development of diabetes secondary to CP.

Diabetes associated with CP may be further distinguished from Type I diabetes by differences in the secretion patterns of pancreatic hormones. Levels of circulating insulin are somewhat diminished in CP, as they are in Type I diabetes, although β-cell function is preserved to a larger extent in diabetes secondary to CP than in age- and duration-matched Type I diabetics (14). Unlike the pattern seen in Type I diabetes, pancreatic polypeptide (PP) and glucagon levels are markedly low, or even absent, in CP and, more generally, in pancreatogenic diabetes (13,22-25). The deficiency of one or more of these pancreatic islet cell hormones in CP may contribute to the impaired glucose tolerance in pancreatitic patients, as discussed below.

Hepatic Insulin Resistance in Chronic Pancreatitis

Insulin resistance is defined as a state in which a normal concentration of insulin produces a less than normal biological response, or as hyperinsulinemia in the presence of normal or elevated blood glucose levels (26,27). Normoglycemia can be maintained in some insulin resistant states by hyperinsulinemia. However, if the pancreatic β-cells are not able to secrete sufficient insulin to compensate, hyperglycemia results. It is generally accepted that all diabetic syndromes share some degree of insulin resistance (27).

The mechanisms by which insulin controls blood glucose levels in the body are extremely complex, and the processes by which it regulates cellular function are largely yet unknown. In general, insulin function depends upon three integrated processes involving its synthesis and secretion from β-cells, its circulation in the bloodstream, and its target cell actions. Thus, insulin resistance can be classified according to the following scheme: (a) abnormal β-cell secretion, (b) altered transport of insulin in the bloodstream and circulating antagonists, and (c) altered action at the target cell level. In very rare cases, a mutated insulin molecule or a hyperproinsulinemic state can present with the clinical features of insulin resistance (27). Autoantibodies to insulin can reduce the access of the molecule to its target cells, and circulating hormonal antagonists, including cortisol, glucagon, and catecholamines, can also produce clinical insulin resistance (27). However, in most patients who present with reduced sensitivity to insulin, the abnormality occurs at the level of target cell action and can be further classified as either a receptor or post-receptor defect (27). The insulin resistance in obesity, in Type II (insulin-independent) diabetes, and in pancreatogenic diabetes, is due to target cell defects.

The liver plays a crucial role in maintaining glucose homeostasis. In the post-prandial state, the liver serves as the principal source of glucose, as the processes of gluconeogenesis and glycogenolysis contribute to total hepatic glucose production. During a meal, insulin is released from the β-cells and suppresses hepatic glucose production. Under normal circumstances, hepatic glucose production is exquisitely sensitive to minimal increases in the level of circulating insulin. A loss of this hepatocyte

sensitivity to insulin has been demonstrated to be a critical factor in the abnormal glucose metabolism in some diabetic patients (28).

In pancreatogenic diabetes, insulin resistance is at the level of the target cell and is specific for the liver (29-31). Several investigators (29,32-35) have utilized euglycemic glucose-clamp experiments to document that hepatic sensitivity to insulin is lost in CP. In this technique, the effects of a continuous insulin infusion on hepatic glucose production and overall glucose disposal are assessed by measuring the turnover of 3-[^{3}H]-

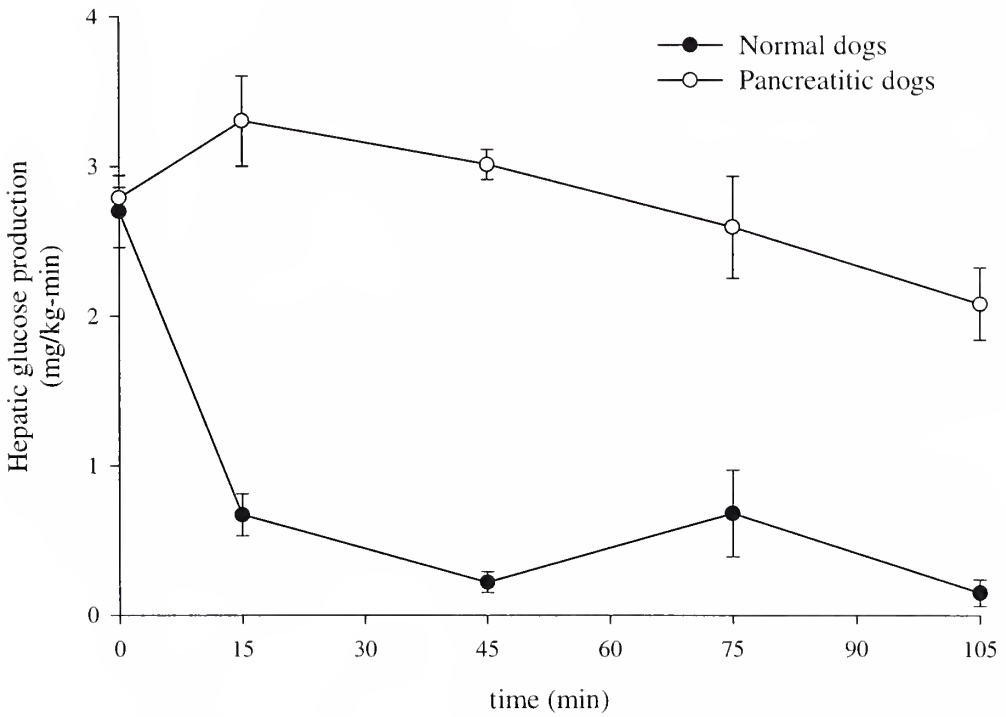


Figure 1. Hepatic glucose production as measured by euglycemic glucose-clamp technique. Normal dogs (*black circles*, $n = 4$) and dogs with chronic pancreatitis (*white circles*, $n = 4$) received a two-hour infusion of insulin (2.0 mU/kg-min). Euglycemia was maintained by variable 20% glucose infusion based on plasma glucose levels obtained every 5 minutes. Hepatic glucose production was calculated from changes in glucose specific activity during fixed infusion of 3-[^{3}H]-glucose. Reproduced from Reference 34.

glucose during the maintenance of euglycemia. As shown in Figure 1, in response to hyperinsulinemia, normal dogs exhibit up to a 95% suppression of hepatic glucose production, while dogs with CP demonstrate only a 30% suppression (34). These findings have indicated that surgically-induced chronic pancreatitis results in the loss of hepatic sensitivity to insulin with consequent persistence of high rates of hepatic glucose production. Inappropriate maintenance of hepatic glucose production despite the presence of exogenously elevated insulin levels has also been demonstrated following pancreatic resection in man (36).

Ex vivo isolated, single-pass, liver perfusion studies have provided the opportunity to examine hepatic sensitivity to insulin in the absence of other extrahepatic factors. As in the euglycemic glucose-clamp experiments, these studies have demonstrated a loss of insulin-mediated inhibition of glucagon-stimulated hepatic glucose production in chronic pancreatitis (30,31). Sham-operated rats demonstrated a dose-dependent insulin-induced suppression of hepatic glucose production (Figure 2), while CP rats showed no response to insulin (30). These results have suggested that chronic pancreatitis is associated with a primary hepatic resistance to insulin resulting from an intrinsic hepatic defect in the regulation of glucose metabolism. Indeed, CP is accompanied by a specific hepatic resistance to insulin, as peripheral—i.e., adipocyte and skeletal muscle cell—glucose uptake remains normal (29). This is in contrast to Type I and Type II diabetes, in which insulin resistance is more generalized, involving adipose tissue and skeletal muscle (27).

Although it is known that abnormal hepatic glucose production contributes to the glucose intolerance seen in chronic pancreatitis, the cellular mechanism of hepatic insensitivity to insulin in CP is yet to be determined. Abnormalities in the availability of

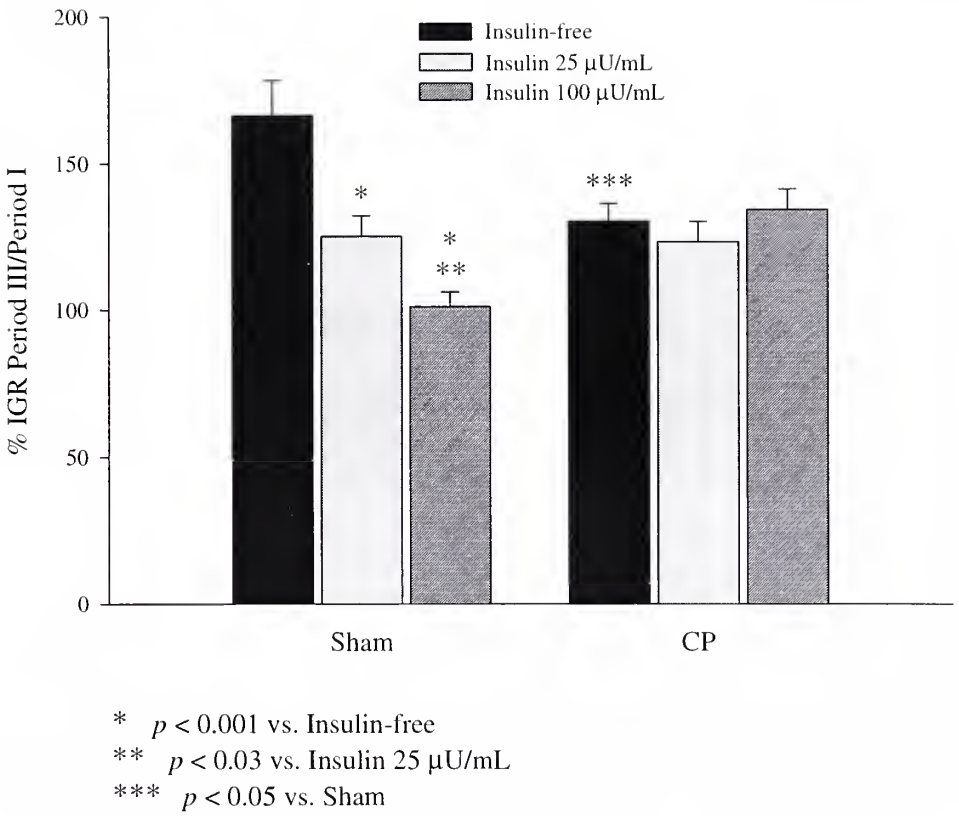


Figure 2. Effect of insulin perfusion on integrated glucose response (IGR) during *ex vivo* isolated liver perfusion in sham rats and in chronic pancreatitic rats (CP). Glucagon was perfused for three continuous 10-minute periods (Periods I, II, III). Insulin was added to infusate after Period I. The insulin dose-response seen in sham livers is absent in CP livers. Reproduced from Reference 29.

the insulin receptor and the facilitated-diffusion glucose transporter, GLUT2, on the hepatocyte plasma membrane in CP may help to elucidate the cellular defects that result in insulin resistance and persistent hepatic glucose production.

Insulin Receptor Protein

Following release from the pancreatic β-cell, insulin effects a reduction in blood glucose by inhibiting hepatic glucose production and by stimulating peripheral glucose utilization. Although the regulation of blood glucose is its most clinically relevant action, insulin also influences protein, carbohydrate, and lipid metabolism, gene transcription, and cellular growth and differentiation (37). As insulin has a multitude of effects in different tissues, as well as within the same cell, the key to understanding its molecular basis of action rests in the investigation of mechanisms of signal transduction. It is well-known that insulin's signaling cascade involves autophosphorylation of its receptor upon ligand binding (38,39) and a subsequent tyrosine phosphorylation cascade of cellular substrates (40-45). Recently, two second messenger molecules, inositolphosphoglycan and diacylglycerol, produced at the plasma membrane have been demonstrated to mediate many of insulin's metabolic effects and insulin's stimulation of glucose transport, respectively (46,47). Evidence has also suggested that insulin may mediate some of its cellular effects by a direct interaction of the hormone with intracellular organelles, such as the nucleus (48,49). Although the molecular mechanisms by which insulin exerts its pleiotropic biologic effects on target cells are complex and require further elucidation, it is well-established that the initial event is the interaction of the hormone with its receptor on the cell membrane (41,50-53).

The insulin receptor (IR) is a heterotetrameric integral membrane glycoprotein composed of two different subunits, the α-subunit (M_r = 135 kDa) and the β-subunit (M_r = 95 kDa). Two extracellular α-subunits are linked to two transmembrane β-subunits by

disulfide bonds to produce the tetrameric structure of IR (43,52,54). The ligand binding site is located on the extracellular α-subunits, and the transmembrane β-subunits contain cytoplasmic tyrosine kinase moieties which initiate the phosphorylation cascade that results in many of insulin's cellular actions (39,42,52,54). Following the binding of insulin to its receptor on the cell surface, the insulin-IR complex undergoes internalization, after which insulin is degraded and IR is recycled directly to the plasma membrane (55-59). Although the major cellular effects mediated by insulin occur when the ligand binds to its cell surface receptor, evidence has suggested that some biological actions may be regulated following internalization of the hormone (48,49,52,60).

The insulin-IR interaction has been studied extensively in several insulin-resistant states to attempt to elucidate the role of the receptor in diminished sensitivity to insulin. Several studies in animal models and in man have demonstrated that obesity results in diminished insulin binding to plasma membrane receptors (61-68). Furthermore, by Scatchard plot analysis, the decreased insulin binding observed in this insulin-resistant state results from a decrease in the number of cell surface insulin receptors—i.e., down-regulation due to elevated circulating insulin—and that affinity for their ligand remains normal (69-71). Similarly, in non-obese Type II diabetic patients, diminished insulin binding in circulating monocytes and in abdominal skeletal muscle results from a reduction in the number of cell surface receptors and correlates with the degree of insulin resistance (72-74). In 1993, Damm et al. demonstrated that decreased insulin binding in skeletal muscle of women with gestational diabetes results from a reduced number of receptors and may play a role in the insulin resistance associated with pregnancy (75). The glucose intolerance and insulin resistance that is associated with glucocorticoid

excess, as in Cushing's syndrome or due to glucocorticoid therapy, has a different mechanism with respect to the insulin-IR interaction. In these states, the diminished insulin binding observed is due to a reduced affinity of IR for its ligand (76), rather than to a decrease in the number of cell surface receptors. Thus, both reduced plasma membrane IR availability and reduced IR affinity have been described as causes of insulin resistance.

Competitive insulin binding studies have recently been performed in livers from rats with chronic pancreatitis to determine whether altered hepatic insulin binding may contribute to the reduced hepatic sensitivity to insulin in CP. As shown in Figure 3, results have indicated that CP is associated with diminished hepatocyte insulin binding and that the altered binding is the result of a decrease in the number of plasma membrane-bound receptors (77). In addition, IR affinity was not found to be altered in CP livers. In further support of the notion that the insulin resistance in CP is specific for the liver, assessment of skeletal muscle insulin binding did not reveal abnormalities in IR availability or affinity in CP (77). These results are in contrast to models of obesity, in which a generalized reduction in IR availability affects skeletal muscle, as well as other tissues (63,65-67,78,79). Although it is well-known that the availability of receptor proteins on the plasma membrane is regulated by the processes of synthesis, degradation, and intracellular trafficking of newly synthesized or internalized proteins, the mechanism that accounts for the reduction in hepatocyte plasma membrane-bound IR in CP requires further investigation.

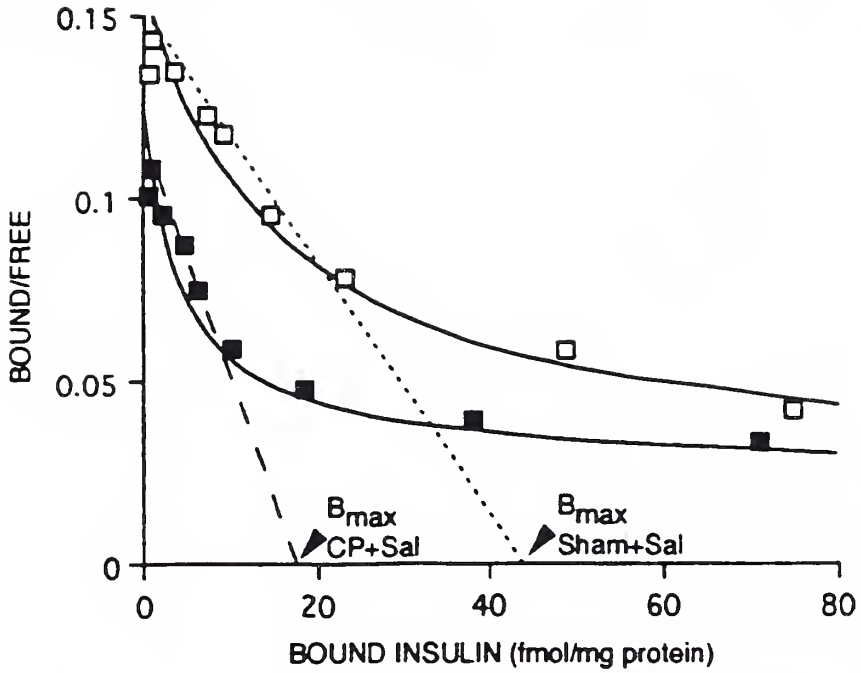


Figure 3. Effects of chronic pancreatitis on hepatic insulin binding. Scatchard plot analysis of insulin binding in solubilized hepatic membranes from sham (*white squares*) and chronic pancreatitic (CP) rats (*black squares*). Abscissa intercept represents maximum binding capacity (B_{max}). Reproduced from Reference 77.

GLUT2 Facilitated-Diffusion Glucose Transporter Protein

The transport of polar glucose molecules across the hepatocyte plasma membrane is regulated by the facilitated-diffusion glucose transporter, GLUT2. GLUT2 is a 52-kDa integral membrane protein encoded on chromosome 3 and found primarily in pancreatic β-cells and in the sinusoidal membrane of hepatocytes (80,81). The molecule consists of five transmembrane domains which form a pore in the membrane and catalyze the transport of glucose down its concentration gradient via an energy-independent

conformational oscillation (82-84). GLUT2 accounts for 90% to 97% of the glucose transporter protein found in hepatocytes (80,85), and over 90% of hepatocyte GLUT2 is present in the cell membrane in the basal state (86). Because of its low affinity, or high K_m (15-20 m*M*), GLUT2 allows the rate of glucose transport to change proportionally as the intracellular or extracellular glucose concentration fluctuates (87).

GLUT2 has been studied most extensively in the pancreatic β-cell, where abnormalities in the level of its membrane availability are believed to play a pathophysiologic role in impaired glucose-stimulated insulin release in models of diabetes (88-90). It has recently been demonstrated in a mouse model of streptozotocin-induced diabetes that a significant reduction in GLUT2 protein and mRNA expression in pancreatic β-cells develops prior to the manifestation of diabetes (91). The reduction in the availability of GLUT2 on the plasma membrane results in a decrease in the rate of glucose transport into the β-cell, further supporting the notion that GLUT2 is a critical factor in the loss of glucose-induced insulin secretion (92).

Although the role of GLUT2 in abnormal hepatic glucose metabolism is less clear, recent studies have suggested that altered GLUT2 availability may contribute to the loss of insulin-mediated suppression of hepatic glucose production in CP. As shown in Figure 4, the insulin-induced reduction of hepatic glucose production observed in normal rats in *ex vivo* isolated liver perfusion experiments is associated with a 30% reduction in the hepatocyte plasma membrane-bound quantity of GLUT2 (31). Furthermore, a complete loss of this insulin-induced change in GLUT2 availability accompanies the persistently high rate of hepatic glucose production in CP (31).

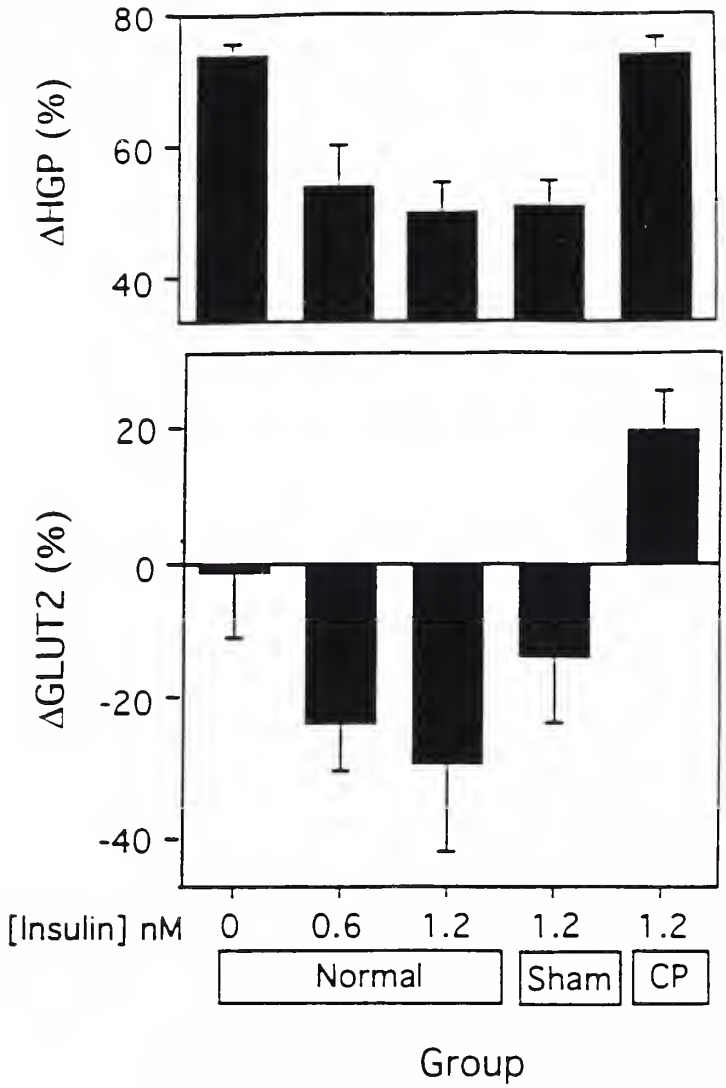


Figure 4. Changes in glucagon-stimulated hepatic glucose production (HGP) and in hepatocyte plasma membrane-bound GLUT2 in normal, sham, and chronic pancreatitic (CP) rats following insulin infusion (0-1.2 n*M*) in *ex vivo* isolated liver perfusion studies. Reproduced from Reference 31.

The regulation of facilitative glucose transporter proteins by insulin has been studied extensively in adipose and skeletal muscle tissue [see Kahn (93) for a review]. In adipocytes and skeletal muscle cells, the transport of glucose across the cell membrane is

regulated by another facilitated-diffusion glucose transporter, GLUT4 (82-84,94). Over 90% of the GLUT4 present in adipocytes is located in cytoplasmic vesicles (95-97), and the presence of insulin causes translocation of the vesicles to the cell surface and the resultant movement of GLUT4 from the intracellular pool to the plasma membrane (95,98,99). By this mechanism, insulin increases the rate of glucose uptake into adipocytes, thereby regulating glucose utilization in the periphery. Similarly, recent studies have shown that the insulin-mediated increase in the quantity of plasma membrane-bound GLUT4 is responsible for the increased rate of glucose uptake in skeletal muscle (100,101).

As described above, 90% of the hepatocyte GLUT2 protein is present in the cell membrane in the basal state. As initial studies failed to demonstrate any further increase in hepatocyte plasma membrane GLUT2 availability, it had previously been thought that insulin has no effect upon GLUT2 distribution in the liver. Our recent finding that a decrease in hepatocyte plasma membrane-bound GLUT2 occurs in normal rat livers during insulin perfusion suggests that, in fact, insulin does effect a change in GLUT2 availability in the hepatocyte. Although the mechanism of this insulin-induced change in GLUT2 is unknown, it may be mediated by accelerated degradation or internalization of the GLUT2 protein. The potential role of GLUT2 in the insulin-induced suppression of hepatic glucose production also requires elucidation. Furthermore, it is uncertain how chronic pancreatitis results in a disruption of these changes in GLUT2 and whether a defect in the regulation of GLUT2 may contribute to the altered hepatic glucose production in CP.

Intracellular Vesicle Trafficking

The availability of receptors and transporter proteins on the plasma membrane is regulated by three concurrent processes: (a) synthesis of new proteins, (b) degradation of old proteins, and (c) intracellular trafficking of newly synthesized or internalized proteins to the plasma membrane. Defects in intracellular vesicle trafficking have been implicated in the altered plasma membrane availability of various receptors in hepatocytes. Evidence has suggested that the reduced number of asialoglycoprotein receptors observed on the hepatocyte plasma membrane of ethanol-fed rats may result from defective receptor recycling (102). IR endocytosis and recycling in hepatocytes involves interaction with a population of intracellular clear vesicles (103), and monensin, an inhibitor of receptor recycling, has been demonstrated to enhance the insulin-induced down-regulation of cell-surface IR (104).

Several monomeric, low molecular weight GTP-binding proteins (GTP-bps; 20-25 kDa) which belong to the *ras*-superfamily have been demonstrated to participate in intracellular vesicle trafficking—more specifically, in the regulation of endocytosis and exocytosis (105-110). Recent studies have implicated the expression of one such GTP-binding protein, rab5, in regulating the rate of endocytosis in rat liver cells (110). Furthermore, *ras*-related GTP-bps have been demonstrated to play critical roles in the pathway of receptor recycling (106,108). In a prior study, we found that the subcellular distribution of low molecular weight GTP-bps is altered in fractionated livers from rats with diminished hepatocyte plasma membrane-bound IR and the loss of insulin-mediated GLUT2 changes associated with CP (unpublished data). These data suggest that a

disturbance in intracellular vesicle trafficking in CP livers may be due to an altered distribution of low molecular weight GTP-bps, which may be a critical factor in the abnormalities in IR and GLUT2 plasma membrane availability in chronic pancreatitis.

Role of Pancreatic Polypeptide Deficiency

Pancreatic polypeptide is a 4.2-kDa, 36-amino acid peptide which is stored in histologically distinct islet cells (PP cells) and secreted in the post-prandial state in a biphasic manner, mediated by both neural and hormonal stimulation (111-113). PP cells are located primarily in the pancreatic head and uncinate process, and it is well-established that patients who have undergone pancreatoduodenectomy or subtotal distal pancreatectomy and patients with severe chronic pancreatitis have impaired PP secretion (13,22,23,35,36,114).

Several early observations suggested a role for PP in the regulation of glucose metabolism. PP plasma levels have been found to be progressively elevated with increasing age, which is often accompanied by some level of glucose intolerance (115). In 1988, Glaser et al. found elevated PP levels in obese individuals exhibiting oral glucose intolerance (116). It is believed that elevated PP levels in obese subjects with glucose intolerance or with advancing age are the result of compensatory hypersecretion of the hormone in states of abnormal glucose homeostasis. Furthermore, Malaise-Lagae et al. have shown that food intake and body weight are reduced in hyperglycemic mice treated with PP (117).

Studies using models of pancreatogenic diabetes have further elucidated the role of PP as a glucoregulatory hormone. In addition to glucose intolerance and hepatic insensitivity to insulin, chronic pancreatitis is associated with a marked deficiency of PP (13,22,23,35). Utilizing a dog model of chronic pancreatitis, Sun et al. demonstrated that a 14-day subcutaneous infusion of PP resulted in improved oral glucose tolerance and in increased hepatic response to insulin in euglycemic glucose-clamp experiments (32). Similar studies in CP patients deficient in PP have shown that PP replacement enhances insulin-induced suppression of hepatic glucose production in chronic pancreatitis (33-35). In 1995, Seymour et al. demonstrated that PP treatment increases hepatocyte plasma membrane IR availability in CP rats to the levels observed in sham animals and improves glucose tolerance (77).

Although these studies have consistently demonstrated an association between PP deficiency and the altered glucose metabolism and reduced hepatic sensitivity to insulin in CP, the mechanism of the interaction between PP and the hepatic action of insulin is still unknown. The presence of specific PP receptors on hepatocytes, and not in peripheral tissues, suggests that a direct mechanism at the hepatocyte may be responsible (118). However, the effects of PP replacement are not immediate but require a more chronic administration of the hormone (29). In addition, PP receptors have been localized to areas of the rat brain which propagate vagal efferent nerve fibers (119). Thus, it is possible that PP exerts its glucoregulatory effects upon the liver via efferent cholinergic pathways. Further studies are required to elucidate the mechanism of PP regulation of hepatic glucose metabolism, though it is likely that PP deficiency in states

of chronic pancreatitis is a critical factor in the hepatic resistance to insulin and the resultant glucose intolerance associated with the disease.

STATEMENT OF PURPOSE

Chronic pancreatitis is associated with impaired glucose tolerance, reduced hepatic sensitivity to insulin, and a loss of insulin receptor availability on hepatocyte plasma membranes. In normal and sham-operated (control) rats, the insulin-induced suppression of hepatic glucose production is associated with a decrease in the quantity of hepatocyte plasma membrane-bound GLUT2. This insulin-mediated response is absent in chronic pancreatitis. The abnormalities in insulin receptor and GLUT2 availability in chronic pancreatitis may be the result of altered intracellular vesicle trafficking of internalized receptor and transporter proteins and may play a role in the hepatic insulin resistance and glucose intolerance associated with chronic pancreatitis.

Specific Aims:

(1) To determine whether hepatocyte endocytosis is altered in a rat model of chronic pancreatitis;

(2) To determine whether hepatocyte GLUT2 is actively internalized in sham rats;

(3) To determine whether the internalization of hepatocyte GLUT2 is impaired in a rat model of chronic pancreatitis.

MATERIALS AND METHODS

Induction of Chronic Pancreatitis

The protocol used for the induction of chronic pancreatitis is a modification by Seymour et al. (30) of the method of Mundlos et al. (120). Six-week old male Sprague-Dawley rats weighing 150-200 g were purchased from Charles River (Raleigh, NC) and conditioned in the Yale Animal Resources Center for 7 to 10 days prior to the start of the study. After this period, rats were anesthetized by intraperitoneal injection (1.0 mL/kg) of sodium thiopental (Abbott Laboratories; North Chicago, IL). A 2.5-cm midline abdominal incision was made, and the distal common bile duct was intubated at its junction with the duodenum. Acute pancreatitis was induced by infusion of 99% oleic acid (Sigma Chemical Co.; St. Louis, MO) into the pancreatic ducts at 12.5 μL/min for 4 minutes, with an additional 4 minutes dwell-time after the infusion. An atraumatic clamp was placed across the proximal common bile duct during infusion to prevent reflux of oleic acid into the biliary system and the liver. Age- and weight-matched control animals underwent sham operation consisting of exteriorization of the duodenal loop and pancreas. Rats were explored for gross confirmation of chronic pancreatitis (Figure 5) prior to sacrifice during subsequent studies. Gross evidence of chronic pancreatitis is evident eight to twelve weeks after the induction of acute pancreatitis.

Approval for the conduct of these studies was provided by the Yale Animal Care and Use Committee at Yale University School of Medicine.

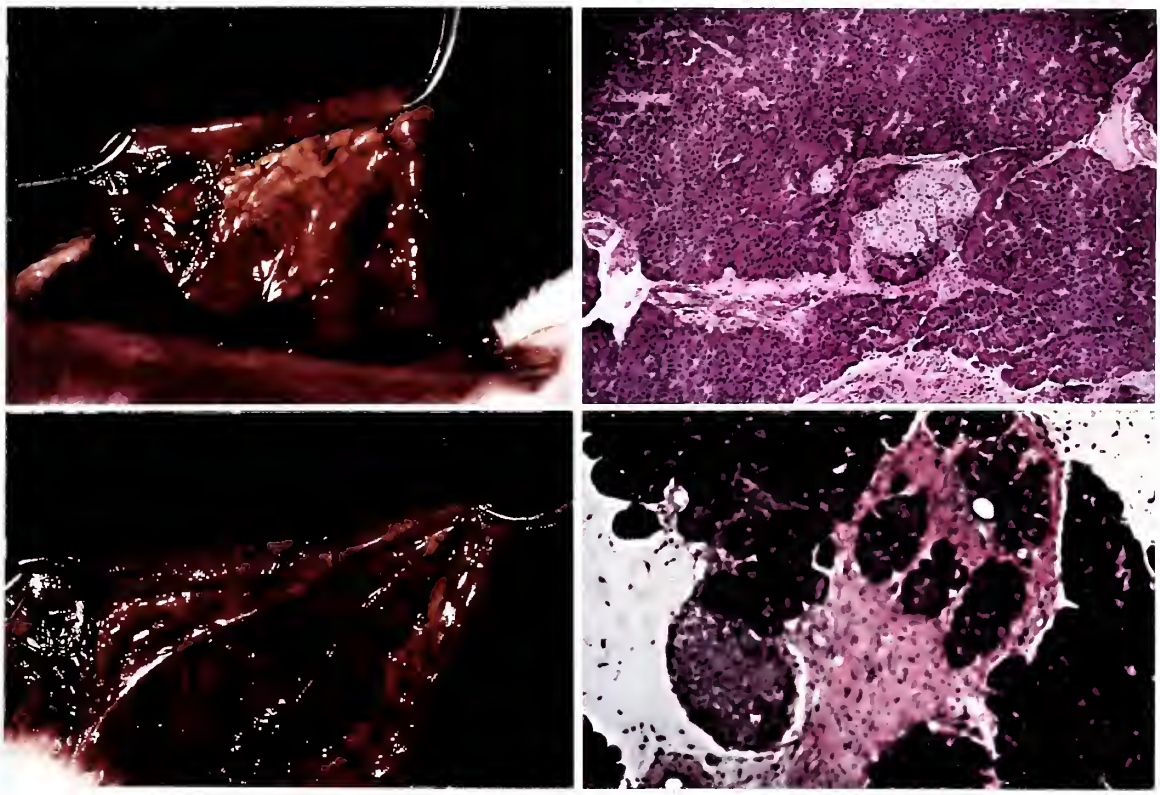


Figure 5. The gross and histologic appearance of the pancreas 8 to 12 weeks after sham operation (top panels). Grossly and histologically, there is no evidence of edema or inflammation. The pancreas appears normal. The bottom panels demonstrate the gross and histologic appearance of the pancreas 8 to 12 weeks after infusion of 50 µl 99% oleic acid into the distal common bile duct. Grossly, there is loss of pancreatic mass, blunting of pancreatic lobules, and replacement of normal pancreatic parenchyma by fibrous tissue. Histologically, there is replacement of exocrine tissue with fat cells and broad areas of fibrosis.

FITC-Dextran Infusion Study Design (Figure 6)

Eight to ten months following induction of pancreatitis, CP and sham rats were either fasted overnight or allowed access to standard chow (Prolab RMH 3000 Short Cut;

PMI Feeds Inc.; St. Louis, MO) *ad libitum*. Following anesthesia by intraperitoneal sodium thiopental (1.0 mL/kg) and cutdown to the right femoral vein, rats received a five-minute intravenous infusion (80 mg/kg) of fluorescein-isothiocyanate labeled 70-kDa dextran (FITC-D; Sigma Chemical Co.; St. Louis, MO), a marker for fluid-phase endocytosis (121-126), by 30-gauge needle. Ten minutes post infusion, livers were

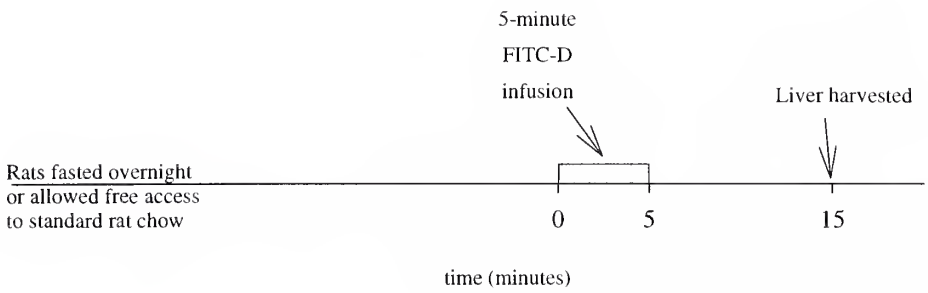


Figure 6. Study design for the measurement of hepatocyte fluid-phase endocytosis in sham and chronic pancreatitic rats utilizing intravenous infusion of 80 mg/kg FITC-Dextran.

removed and minced into 1- to 2-mm specimens. Specimens were fixed in 4% paraformaldehyde for two hours, rinsed in PBS (pH 7.4), and embedded in Tissue-Tek OCT Compound (Miles Laboratories; Elkhart, IN). Specimens were sectioned at 10 μm thickness using a Reichert-Jung 2800 Frigocut E cryostat (M.O.C. Inc.; Valley Cottage, NY), and sections were thaw-mounted onto gelatin-coated slides. Seventy percent glycerol in PBS was applied, and coverslips were sealed with nailpolish. Sections were examined using a Biorad MRC-610 confocal laser scanning microscope (Biorad Microscience Division; Cambridge, MA). FITC-D uptake was determined by mean pixel

intensity, expressed as intensity units (IU), in six random, 1000x fields in each of 3 rats in each group.

FITC-Dextran Time-Course Study Design (Figure 7)

Six months following induction of pancreatitis, fasting CP and fasting sham rats were anesthetized using intraperitoneal sodium thiopental (1.0 mL/kg). Following ventral midline incision, a liver biopsy specimen was taken as a control for background liver fluorescence, with hemostasis attained by securing a 0-silk suture proximal to the biopsy site. A 2-mm gastrotomy incision was made in the anterior gastric wall one centimeter proximal to the pylorus, and the proximal duodenum was intubated with a flexible rubber catheter (0.08 cm ID, 0.24 cm OD; Norton Co.; Akron, OH). With the pylorus manually clamped, a duodenal feeding bolus of 0.5 mL/100 g of a liquid meal (1 kcal/mL; 14% protein, 53% carbohydrate, 33% fat; NuBasics; Clintek; Deerfield, IL) was delivered. Control animals received duodenal intubation alone. Ten minutes post duodenal feeding or intubation alone, a five-minute femoral vein infusion of FITC-D (80 mg/kg) was begun. Sequential liver specimens were harvested at 10, 20 and 30 minutes post femoral vein infusion. Following each harvest, a 0-silk suture was tied proximally around the biopsied liver lobe to attain hemostasis. A standard pre-determined sequence of biopsy sites was followed in all animals. Specimens were minced, fixed, embedded and sectioned as described for the preceding FITC-Dextran Infusion Study. Specimens were examined using an LSM 410 confocal laser scanning microscope (Zeiss; Germany).

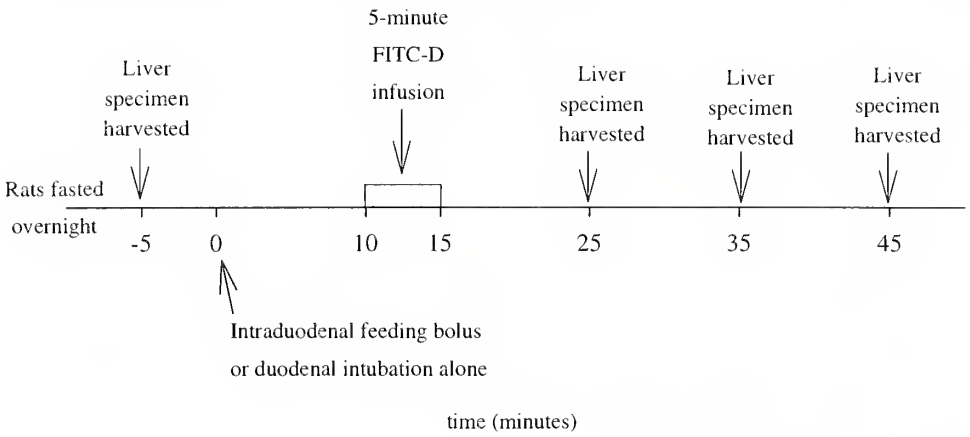


Figure 7. Study design for the time-course measurement of hepatocyte fluid-phase endocytosis in sham and chronic pancreatitic rats utilizing intravenous infusion of 80 mg/kg FITC-Dextran.

FITC-D uptake was determined by mean pixel intensity (IU) in five random, 800x fields per time-point in each of 5 rats in each group.

GLUT2 Study Design

Two to three months following induction of pancreatitis, fasting CP and fasting sham rats were anesthetized using intraperitoneal sodium thiopental (1.0 mL/kg). As described for the FITC-Dextran Time-Course Study, a 2-mm gastrotomy incision was made, the proximal duodenum was intubated, and a duodenal feeding bolus of 0.5 mL/100 g of a liquid meal (1 kcal/ml; 14% protein, 53% carbohydrate, 33% fat; NuBasics; Clintek; Deerfield, IL) was delivered. Control animals received duodenal

intubation alone. Forty-five minutes post duodenal feeding or intubation alone, livers were harvested and a small biopsy was minced and fixed in 4% paraformaldehyde for confocal laser scanning microscopy studies, while the remainder was processed and homogenized at 4°C for subcellular fractionation and Western blotting as described below.

Fractionation of Liver Specimens

The subcellular fractionation protocol utilized was developed by the Department of Cell Biology at Yale University School of Medicine (127-129). Following harvest, livers were rinsed in Lactated Ringer's solution, minced into small pieces, and mechanically homogenized in 15 mL homogenization buffer (0.25 *M* sucrose, 10 m*M* triethanolamine, 10 m*M* acetic acid pH 7.4, 1 m*M* EDTA, 1 m*M* PMSF, 1 m*M* benzamidine, 1 m*M* DTT) with eight strokes of a motor-driven Teflon pestle in a glass homogenization tube to obtain > 80% cell lysis leaving nuclei intact. The crude homogenate was centrifuged at 1000 x g at 4°C for 10 minutes (Rotor SS-34, Sorvall RC 5C Plus; DuPont; Wilmington, DE). The pellet (crude nuclear fraction) was resuspended in 5 mL homogenization buffer, and the post-nuclear supernatant was layered onto a 0.15-mL sucrose cushion (1.0 *M* sucrose in TEA buffer) and centrifuged at 100,000 x g at 4°C for 45 minutes (Rotor AH-650, Sorvall UltraPro 80; DuPont; Wilmington, DE). The supernatant (cytosolic fraction) was removed, and the microsomal pellet was resuspended with sucrose/TEA buffer to a final volume of 3 mL and a final concentration of 1.3 *M* sucrose/TEA. The microsomal fraction was transferred to an AH-650 tube and overlaid

with 1 mL 1.1 *M* sucrose and 1 mL 0.6 *M* sucrose (both in TEA buffer) and centrifuged at 133,000 x g at 4°C for 60 minutes. The endosome (E)-enriched fraction was collected from the 1.1 *M*/0.6 *M* sucrose interface, and the plasma membrane (PM)-enriched fraction was collected from the 1.3 *M*/1.1 *M* sucrose interface.

Fraction Enrichment Assays

Fractionation protocol reproducibility and fraction enrichment were confirmed prior to proceeding to the Western blotting study. PM-fraction enrichment was confirmed using an enzyme assay to determine the distribution of the plasma membrane marker 5'-nucleotidase (Sigma Diagnostics; St. Louis, MO). 5'-nucleotidase activity was measured using a DU-64 Spectrophotometer (Beckman Instruments, Inc.; Irvine, CA) and expressed as activity (U) per milligram of protein in each fraction, as determined by the Bradford method (130). E-fraction enrichment was confirmed by fluorimetric analysis of subcellular fractions from normal rats which received a 5-minute femoral vein infusion of FITC-D (80 mg/kg), as a marker of fluid-phase endocytosis. Ten minutes post FITC-D infusion, livers were harvested, minced, and homogenized, and subcellular fractionation was performed as described above. Fractions were diluted 1:100 with 0.25 *M* sucrose/TEA, and fluorescence intensity was measured using an F-2000 Fluorescence Spectrophotometer (Hitachi Instruments, Inc.; Danbury, CT). Background liver fraction fluorescence was similarly determined in subcellular fractions from normal rats which did not receive an intravenous infusion of FITC-D. FITC-D fluorescence intensity was

expressed as intensity units (IU) per milligram of protein in each fraction after subtracting the background liver fraction fluorescence intensities.

Western Blotting Study for GLUT2

Sample buffer (5x; 1.5 *M* Tris-HCl pH 6.8, 50% glycerol, 5% β-mercaptoethanol, 20% SDS, 0.002% bromophenol blue) was added to each sample in a 1:4 (sample:buffer) ratio. Protein concentrations were determined by the Bradford method (130), and the final protein concentration of each sample was adjusted with 1x sample buffer to 1 mg/mL. Endosome-enriched and plasma membrane-enriched fractions from sham and from CP animals in fasting (n = 8,8) and fed (n = 8,8) states were examined. Samples were loaded (20 μg/lane) onto 12.5% SDS-polyacrylamide gels. Prestained low range molecular weight standard (Bio-Rad Laboratories; Hercules, CA) was loaded onto each gel, and crude homogenate from one fed sham rat was similarly loaded (20 μg/lane) onto all gels to serve as an internal control. Following SDS-PAGE, proteins were electrotransferred to Hybond^{TM}-ECL nitrocellulose membranes (Amersham Corp.; Arlington Heights, IL) at 4°C overnight. Membranes were incubated in blocking buffer (5% non-fat dry milk in TBS/0.1% Tween-20) for 60 minutes at room temperature, followed by incubation for 60 minutes with rabbit anti-rat GLUT2 antibody (1:50,000; Biogenesis Inc.; Sandown, NH). Membranes were rinsed with PBS and incubated for 60 minutes with peroxidase-labeled anti-rabbit IgG secondary antibody (1:1000; Amersham Corp.; Arlington Heights, IL), and detection of GLUT2 was achieved by an electrochemiluminescence method (Amersham Corp.; Arlington Heights, IL).

Membranes were exposed to Kodak X-OMAT AR film (Eastman Kodak Co.; Rochester, NY) for 5-30 seconds and developed.

Scanning Densitometry

GLUT2 was quantified by densitometric scanning analysis of exposed and developed films using Personal Densitometer and ImageQuaNT^{TM} software (Molecular Dynamics; Sunnyvale, CA). GLUT2 corresponded to bands of increased optical density at the expected molecular weight (52 kDa) relative to the known low molecular weight standards. Data was obtained by volume quantitation using a rectangular-shaped area-of-interest, and the relative quantity of GLUT2 was expressed as optical density units per milligram of total hepatic protein normalized to the internal control on each blot.

Confocal Microscopy Study for GLUT2

Minced liver specimens were fixed, rinsed, embedded, and sectioned at 10 μm thickness as described above for FITC-Dextran studies. Following thaw-mounting onto gelatin-coated slides, sections were incubated for 60 minutes with blocking solution (0.5% goat serum in PBS/0.7% Triton X-100) at room temperature, and then stained with rabbit anti-rat GLUT2 antibody (1:50; Biogenesis Inc.; Sandown, NH) for 120 minutes. Following rinses with PBS, sections were incubated with rhodamine-labeled anti-rabbit IgG secondary antibody (1:20; Amersham Corp.; Arlington Heights, IL) for 30 minutes. Sections were rinsed, 70% glycerol in PBS was applied, and coverslips were sealed with

nailpolish. Sections were examined using a digital confocal laser scanning microscope (VayTek, Inc.; Fairfield, IA). GLUT2 content was quantified by mean pixel intensity (IU) in an 8 x 16-pixel area of plasma membrane and a 16 x 16-pixel area of cytosol (CYT) in each of 30 random cells/field (400x) in each of three rats per group.

Statistical Analysis

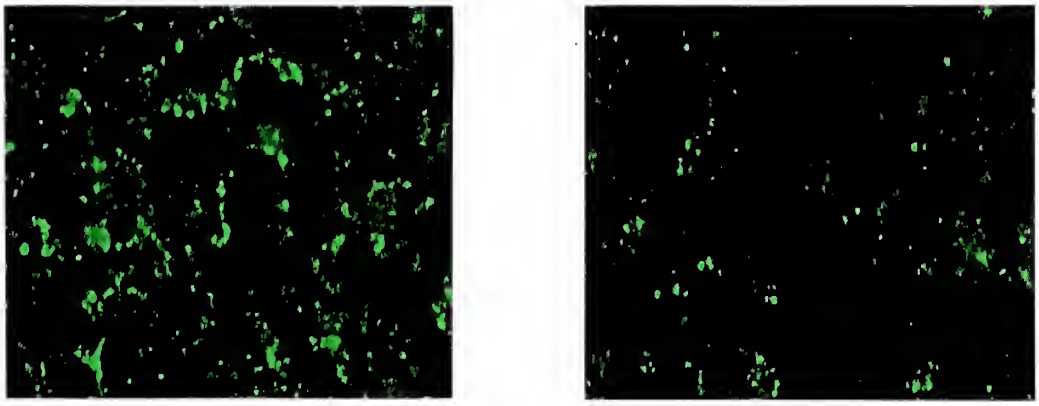
All results are expressed as mean ± SEM. Statistical comparisons between groups were made using the Student's unpaired *t* test. Statistical significance was taken at $p < 0.05$.

All experiments heretofore were conducted by Jaimie D. Nathan. The induction of chronic pancreatitis and the liver harvests were performed with the assistance of Peter D. Zdankiewicz, M.D., and Jinping Wang, M.D.

RESULTS

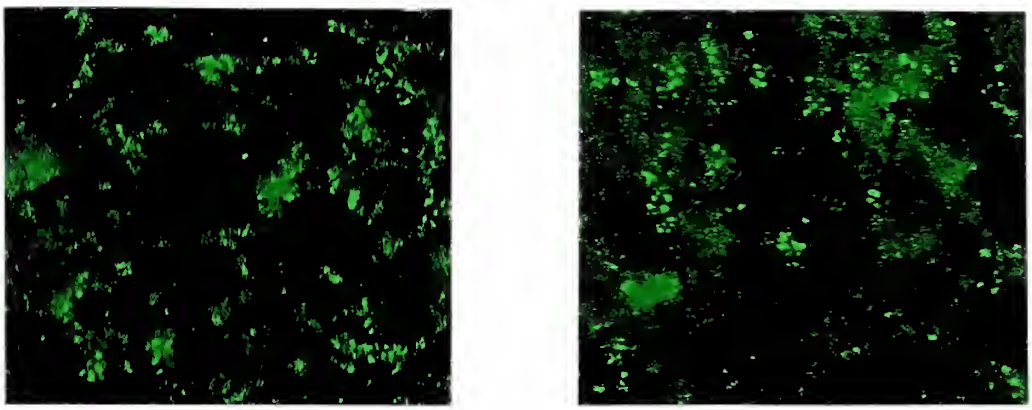
FITC-Dextran Infusion Study

An intravenous infusion of FITC-D was used to measure fluid-phase endocytosis in rat liver *in vivo*. Confocal microscopic images of FITC-D uptake by hepatocytes from



Sham, fasting **Sham, fed**

Figure 8. FITC-Dextran uptake by hepatocytes of sham rats in fasting (*left*) and fed (*right*) states, as determined by confocal laser scanning microscopy (1000x).



CP, fasting **CP, fed**

Figure 9. FITC-Dextran uptake by hepatocytes of chronic pancreatitic (CP) rats in fasting (*left*) and fed (*right*) states, as determined by confocal laser scanning microscopy (1000x).

sham and CP rats in fasting and fed states are shown in Figures 8 and 9. FITC-D uptake was readily apparent in livers from fasting sham rats (56.9 ± 5.9 IU; $n = 3$) but was markedly less in livers from fed sham rats (33.9 ± 1.8 IU; $n = 3$; $p < 0.001$; Figure 10). Livers of fasting CP rats also exhibited active uptake (67.3 ± 5.6 IU; $n = 3$) of FITC-D. However, uptake decreased insignificantly in CP livers from rats allowed *ad libitum* food intake overnight (54.1 ± 4.9 IU; $n = 3$; p = NS; Figure 10). No statistically significant difference was found in FITC-D uptake between fasting sham livers and fasting CP livers.

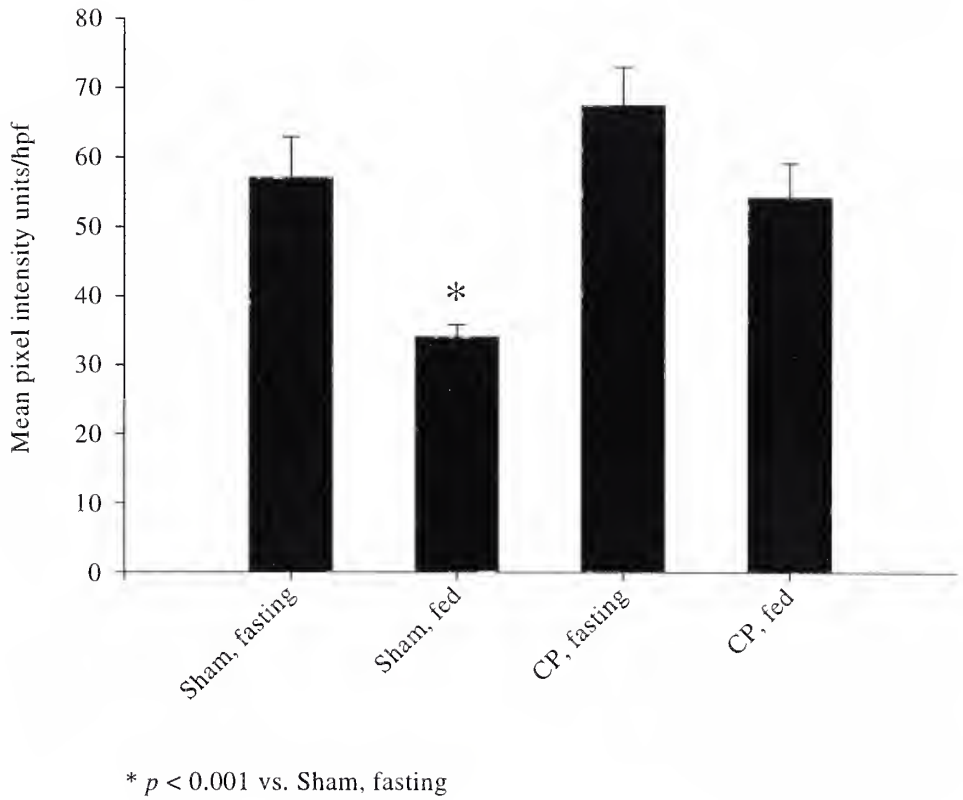
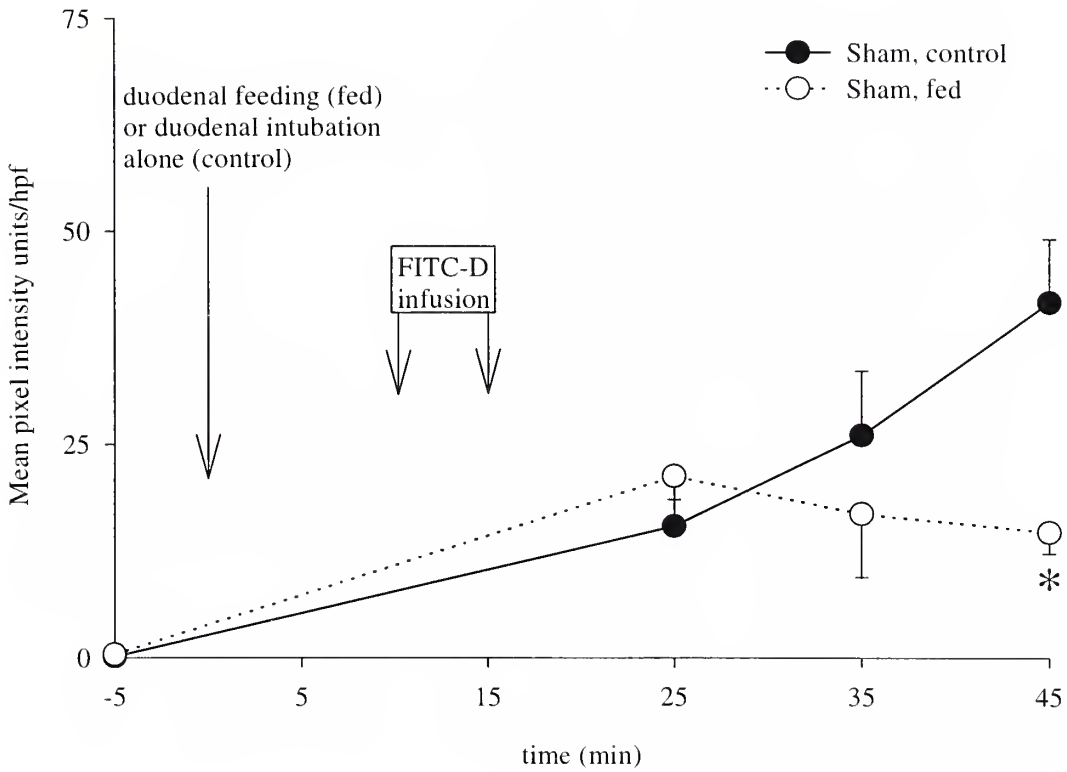


Figure 10. *In vivo* FITC-Dextran uptake by hepatocytes of sham and chronic pancreatitic (CP) rats in fasting and fed states. FITC-D uptake was evident in livers from fasting sham rats but was markedly less in livers from fed sham rats. Livers of fasting CP rats also showed active uptake, which decreased insignificantly in fed CP livers.

FITC-Dextran Time-Course Study

A model recapitulating feeding was used to determine the time-course of the feeding-induced changes in hepatocyte FITC-D uptake observed in the prior study. Intubation of the duodenum was achieved via a gastrotomy incision, and feeding was administered as a bolus. Control rats received duodenal intubation alone. In control



* $p < 0.05$ vs. Sham, control

Figure 11. *In vivo* FITC-Dextran uptake by sham rat livers either following duodenal feeding (fed) or duodenal intubation alone (control). In control sham hepatocytes (*black circles*), FITC-D uptake increased sharply from 25 to 45 minutes post intubation. Following duodenal feeding (*white circles*), uptake remained low in sham rats livers.

sham rats (n = 5), hepatocyte FITC-D uptake increased sharply and linearly with time from 15.4 ± 3.1 IU at 25 minutes post duodenal intubation to 41.6 ± 7.4 IU at 45 minutes ($p < 0.01$ vs. 25 minutes; Figure 11). Following duodenal feeding (n = 5), uptake remained low in sham rats and ranged from 21.1 ± 5.8 IU at 25 minutes to 14.7 ± 2.5 IU at 45 minutes ($p < 0.05$ vs. control sham rats at 45 minutes; p = NS vs. fed sham rats at 25 minutes; Figure 11).

In control CP rats (n = 5), FITC-D uptake trended upward from 21.1 ± 5.5 IU at 25 minutes post duodenal intubation to 31.3 ± 6.8 IU at 45 minutes (p = NS vs. 25

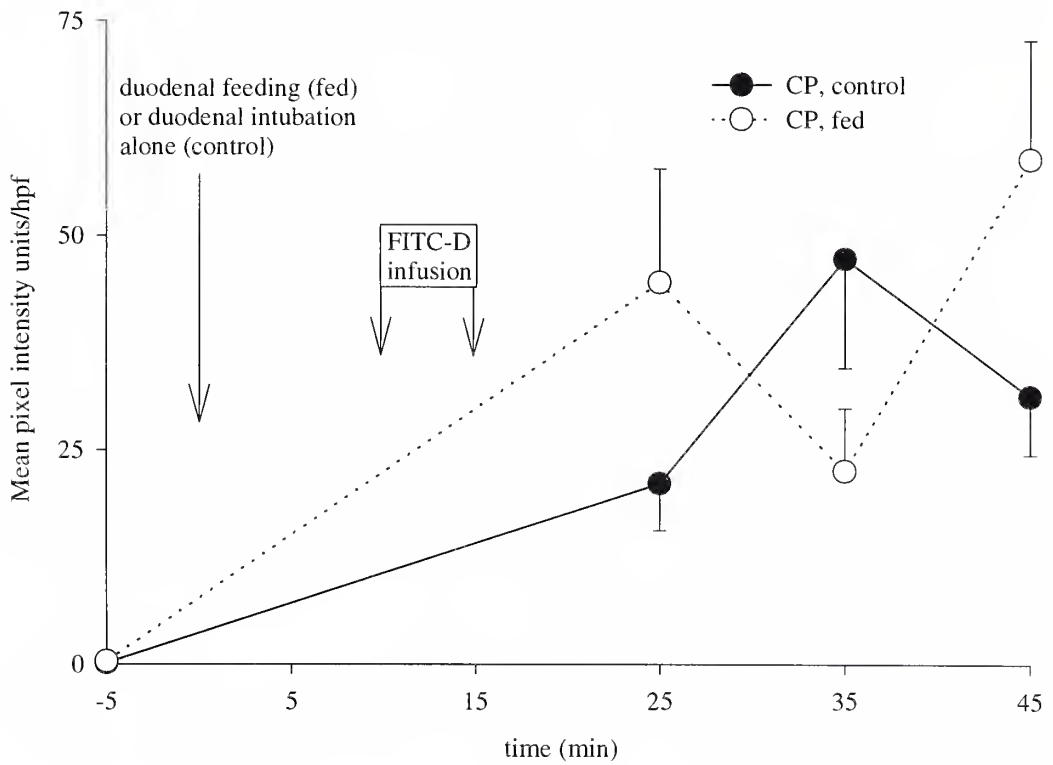


Figure 12. *In vivo* FITC-Dextran uptake by chronic pancreatitic (CP) rat livers either following duodenal feeding (fed) or duodenal intubation alone (control). Between 25 and 45 minutes, hepatocyte FITC-D uptake trended upward both in control CP rats (*black circles*) and in CP rats that received duodenal feeding (*white circles*).

minutes; Figure 12). Uptake also trended upward following duodenal feeding in CP (n = 5), from 44.5 ± 13.3 IU at 25 minutes to 58.9 ± 13.8 IU at 45 minutes (p = NS vs. control CP rats at 45 minutes; p = NS vs. fed CP rats at 25 minutes; Figure 12).

Fraction Enrichment Assays

Subcellular fractionation protocol reproducibility and fraction enrichment were confirmed prior to studying the GLUT2 content of liver fractions from sham and CP rats.

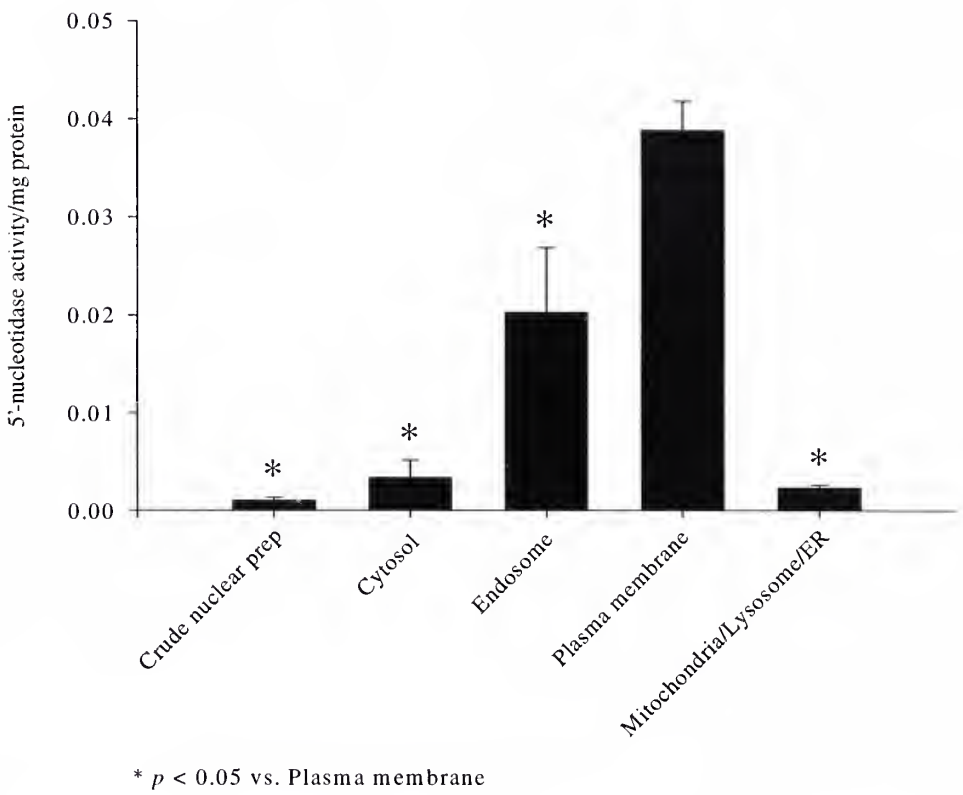


Figure 13. 5'-nucleotidase assay used to determine plasma membrane enrichment of liver fractions. The mean enzyme activity in the plasma membrane (PM)-fraction was approximately 2-times that in the endosome fraction, thereby confirming the relative enrichment of the PM-fraction.

Plasma membrane enrichment was measured using an assay for the plasma membrane marker 5'-nucleotidase. As shown in Figure 13, the mean 5'-nucleotidase activity in the PM-fraction was 0.039 ± 0.003 U/mg, as compared to 0.020 ± 0.007 U/mg in the E-fraction ($n = 3$; $p < 0.05$), an enrichment ratio of approximately 2:1 (PM:E). Endosome enrichment was determined by FITC-D fluorescence intensity following intravenous infusion of the marker of fluid-phase endocytosis. The mean fluorescence intensity in the E-fraction was 5280 ± 610 IU/mg, as compared to 950 ± 280 IU/mg in the PM-fraction ($n = 3$; $p < 0.005$; Figure 14), an enrichment ratio of approximately 5.5:1 (E:PM).

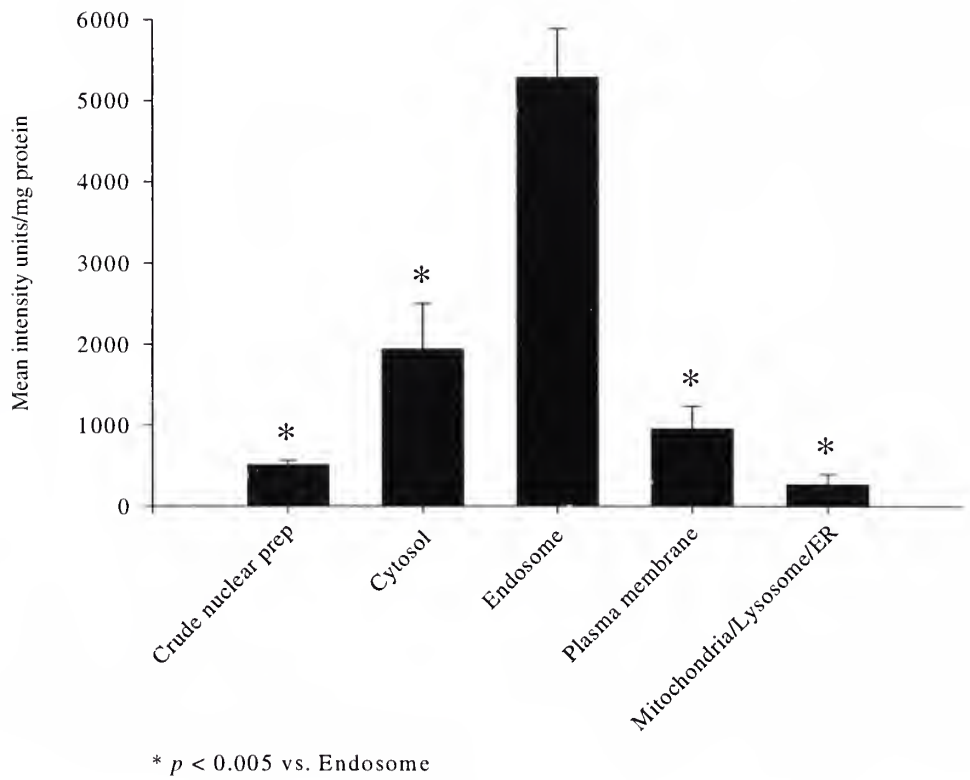


Figure 14. FITC-Dextran fluorescence intensities used to determine endosome enrichment of liver fractions. The mean FITC-D fluorescence intensity of the endosome (E)-fraction was approximately 5.5-times that of the plasma membrane fraction, thereby confirming the relative enrichment of the E-fraction.

Western Blotting Study for GLUT2

Representative Western blots used to quantify GLUT2 in E- and PM-enriched liver fractions from sham and CP rats are shown in Figures 15 and 16, respectively. As

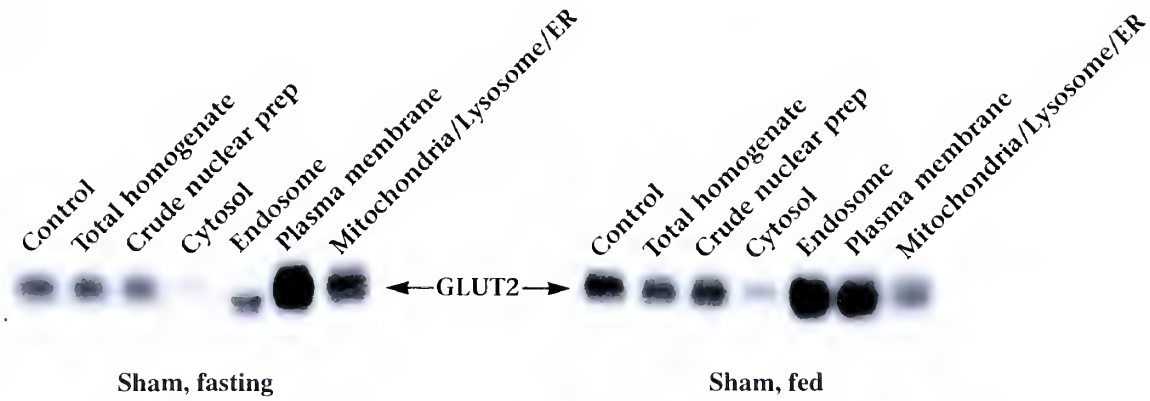


Figure 15. Representative Western blots used to quantify GLUT2 in endosome- and plasma membrane-enriched liver fractions from sham-operated (sham) rats in fasting (*left*) and fed (*right*) states. Feeding induced an obvious increase in the quantity of GLUT2 in the endosome-enriched fraction, as compared to fasting sham rats. GLUT2 content was determined by scanning densitometry. Control = total liver homogenate from sham, fed rat.

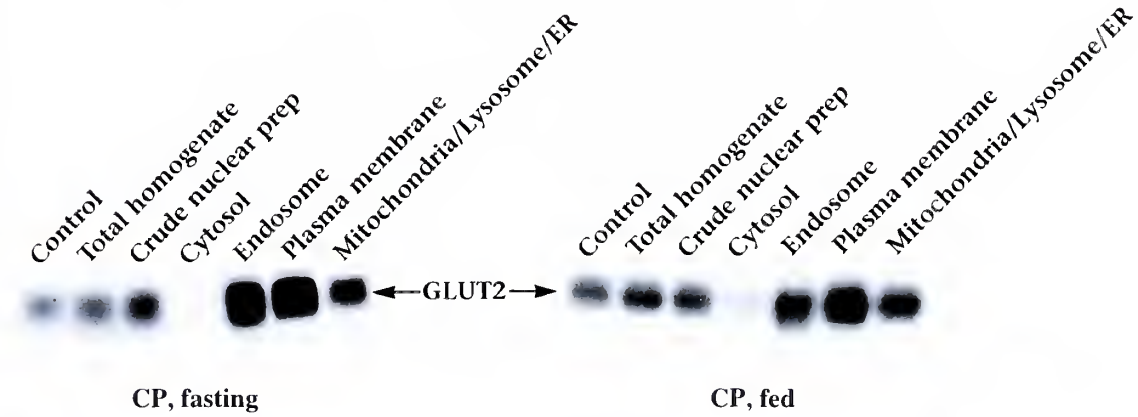
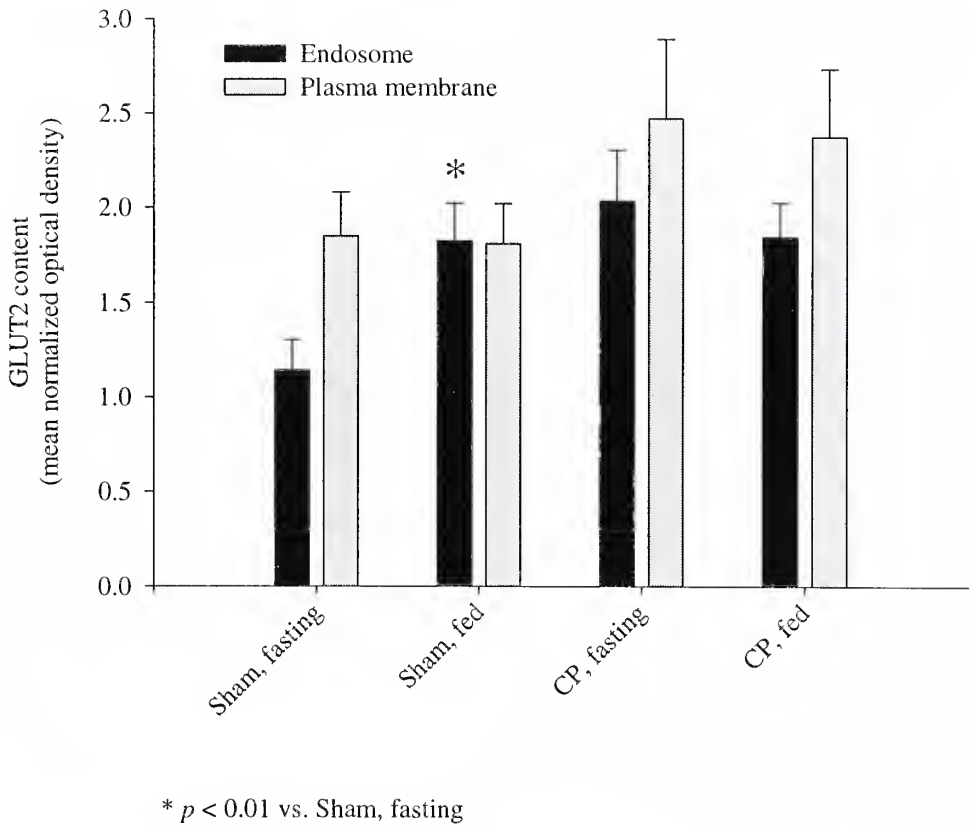


Figure 16. Representative Western blots used to quantify GLUT2 in endosome- and plasma membrane-enriched liver fractions from chronic pancreatitic (CP) rats in fasting (*left*) and fed (*right*) states. GLUT2 quantity of endosome- and plasma membrane-enriched fractions remained unchanged after feeding. GLUT2 content was determined by scanning densitometry. Control = total liver homogenate from sham, fed rat.

demonstrated on the immunoblots, a wide band of increased optical density representing GLUT2 was evident at approximately 52 kDa relative to the low molecular weight standards. The GLUT2 content of the E-enriched and PM-enriched fractions was determined by scanning densitometry and volume quantitation analysis utilizing a rectangular-shaped area-of-interest. The mean normalized GLUT2 content of the E-enriched fractions increased from 1.14 ± 0.16 in fasting sham rats ($n = 8$) to 1.82 ± 0.20 following feeding ($n = 8$; $p < 0.01$), a 60% increase, while there was no statistically



* $p < 0.01$ vs. Sham, fasting

Figure 17. GLUT2 content of endosome-enriched and plasma membrane-enriched liver fractions from sham and chronic pancreatitic (CP) rats after duodenal feeding (fed) or duodenal intubation alone (fasting). Results are expressed as mean normalized optical density, as determined by scanning densitometry and volume quantitation analysis.

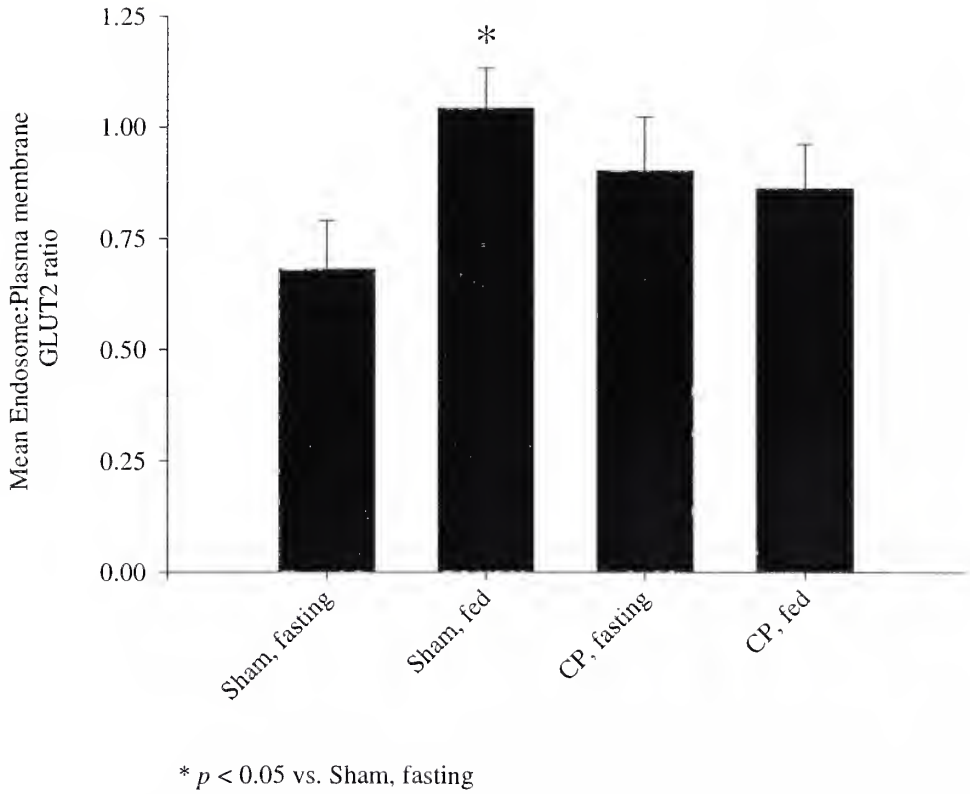


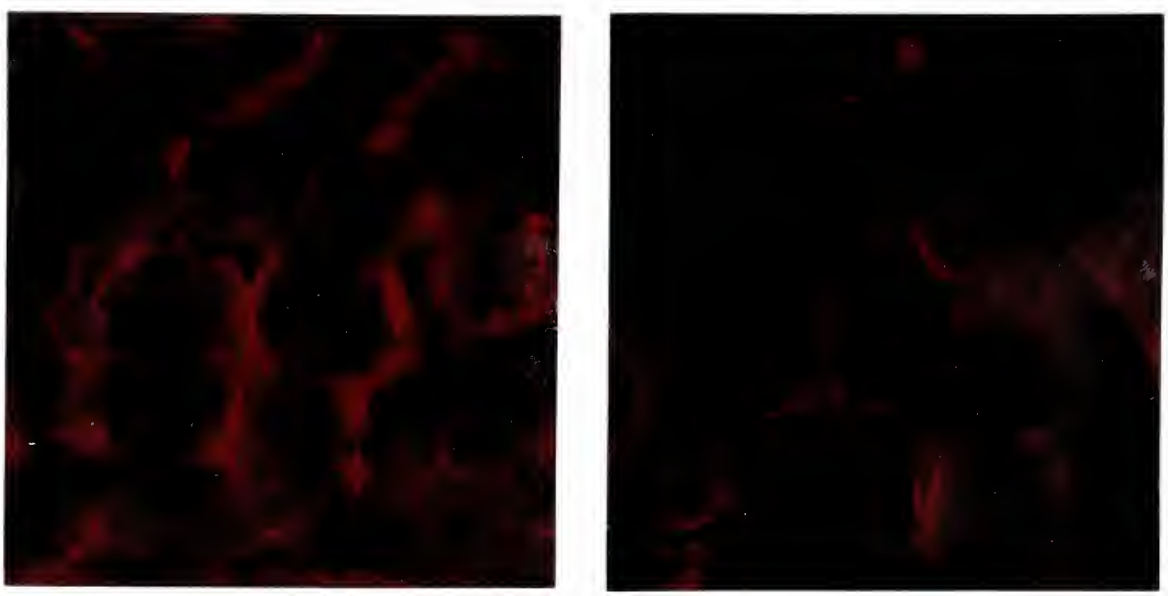
Figure 18. Endosome (E)-to-plasma membrane (PM) ratio of GLUT2 in sham and chronic pancreatitic (CP) rat livers, as determined by scanning densitometry. The E:PM ratio of GLUT2 increased from 0.68 ± 0.11 (mean ± SEM) in fasting sham livers to 1.04 ± 0.09 in fed sham livers ($p < 0.05$). There was no change in the E:PM ratio of GLUT2 in CP livers following duodenal feeding.

significant difference in the GLUT2 content of the PM-enriched fractions between the sham fasting and the sham fed groups (Figure 17). The E:PM ratio of GLUT2 increased from 0.68 ± 0.11 in fasting sham livers to 1.04 ± 0.09 in fed sham livers ($p < 0.05$; Figure 18), an increase of approximately 53%.

The mean normalized GLUT2 content of the E-enriched and PM-enriched fractions from the CP rats in the fasting state were statistically indistinguishable from the respective fractions from the CP rats that were fed a duodenal meal (Figure 17). Furthermore, there was no change in the E:PM ratio of GLUT2 in CP livers following duodenal feeding (0.90 ± 0.12 vs. 0.86 ± 0.10; $n = 8,8$; p = NS; Figure 18).

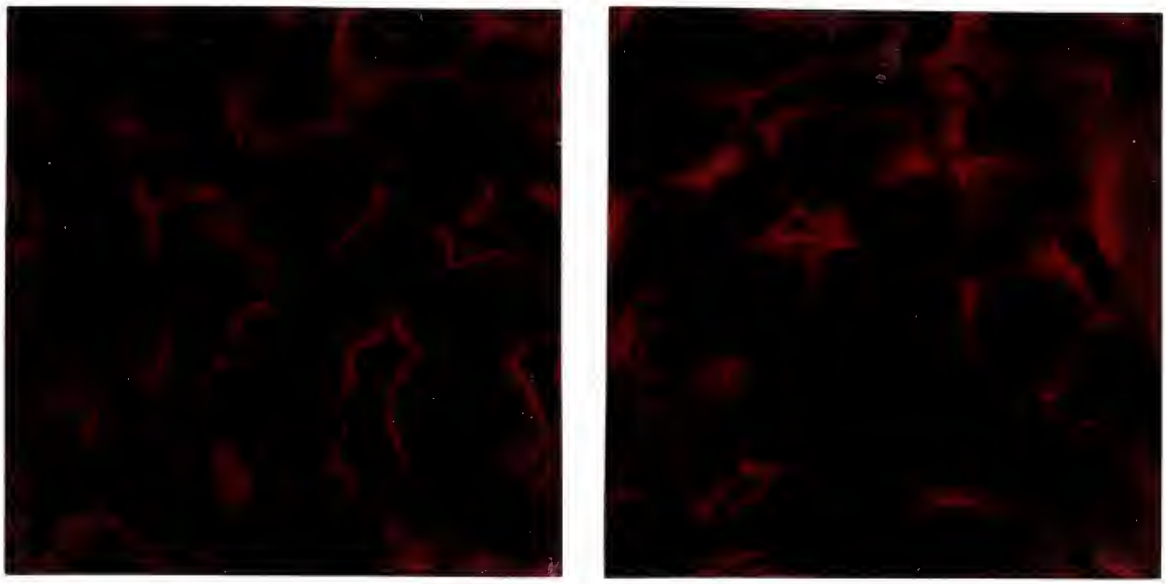
Confocal Microscopy Study for GLUT2

Confocal laser scanning microscopy images of GLUT2 in hepatocytes from sham and CP rats in the fasting state and following feeding are shown in Figures 19 and 20. For the quantification of GLUT2 content in the rat hepatocytes, a 16 x 16-pixel area of cytosol (CYT) was used for the determination of non-plasma membrane bound GLUT2



Sham, fasting **Sham, fed**

Figure 19. Immunofluorescence of GLUT2 in hepatocytes of sham rats in fasting (*left*) and fed (*right*) states, as determined by digital confocal laser scanning microscopy (400x). It is evident that plasma membrane GLUT2 immunofluorescence in fasting sham rat hepatocytes is reduced following feeding.



CP, fasting **CP, fed**

Figure 20. Immunofluorescence of GLUT2 in hepatocytes of chronic pancreatitic (CP) rats in fasting (*left*) and fed (*right*) states, as determined by digital confocal laser scanning microscopy (400x). Cytosolic and plasma membrane immunofluorescence intensities appear unchanged in CP rat hepatocytes after feeding.

and an 8 x 16-pixel area of plasma membrane (PM) was used for GLUT2 bound to the cell surface. As is evident from the study in the sham animals, feeding induced a reduction in the quantity of GLUT2 that was bound to the plasma membrane, from a mean pixel intensity of 2489 ± 44 IU in sham fasting rats (n = 3) to 2154 ± 43 IU in sham fed rats (n = 3; $p < 0.0001$; Figure 21), a decrease of approximately 13%. There was no statistically significant change in the cytosolic GLUT2 in sham fasting rats following feeding. Duodenal feeding increased the CYT:PM ratio of GLUT2 from 0.75 ± 0.01 in fasting sham rat livers to 0.86 ± 0.01 in fed sham rat livers ($p < 0.0001$; Figure 22), an increase of approximately 15%.

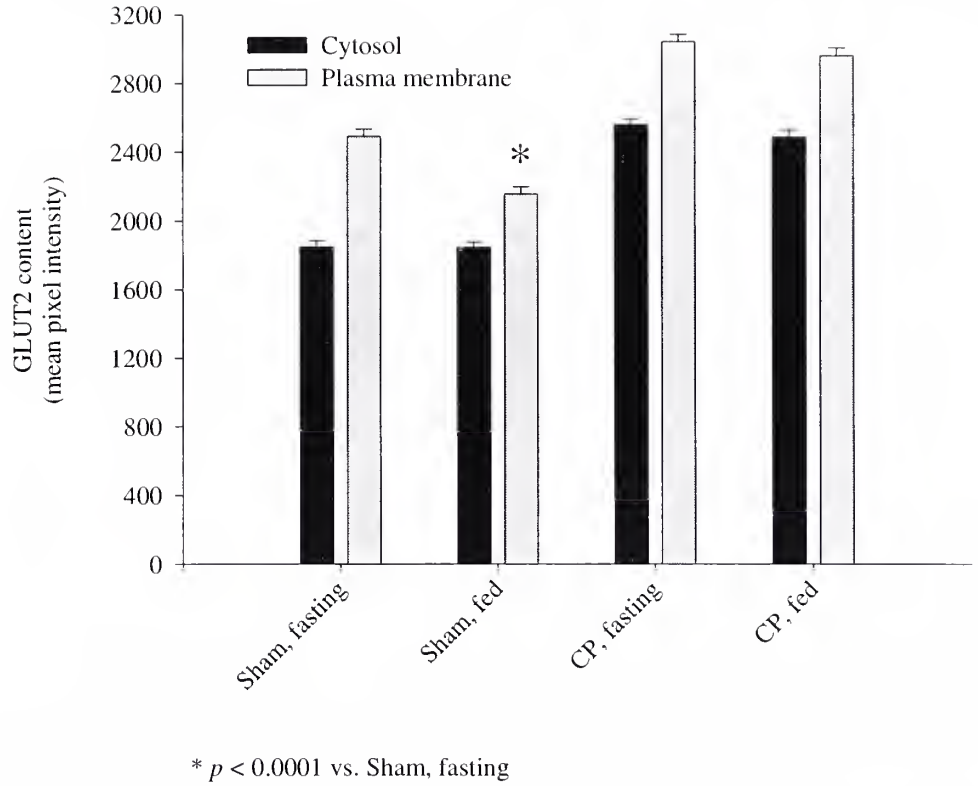


Figure 21. Hepatocyte GLUT2 immunofluorescence in cytosolic and plasma membrane compartments in sham and chronic pancreatitic (CP) rats after duodenal feeding (fed) or duodenal intubation alone (fasting). Results are expressed as mean pixel intensity, as determined by digital confocal laser scanning microscopy.

The mean pixel intensities of CYT and PM GLUT2 in hepatocytes from fed CP rats were statistically indistinguishable from fasting CP rat livers (Figure 21). The CYT:PM ratio of GLUT2 in fasting CP livers (0.84 ± 0.01; $n = 3$) remained unchanged following feeding (0.84 ± 0.01; $n = 3$; p = NS; Figure 22).

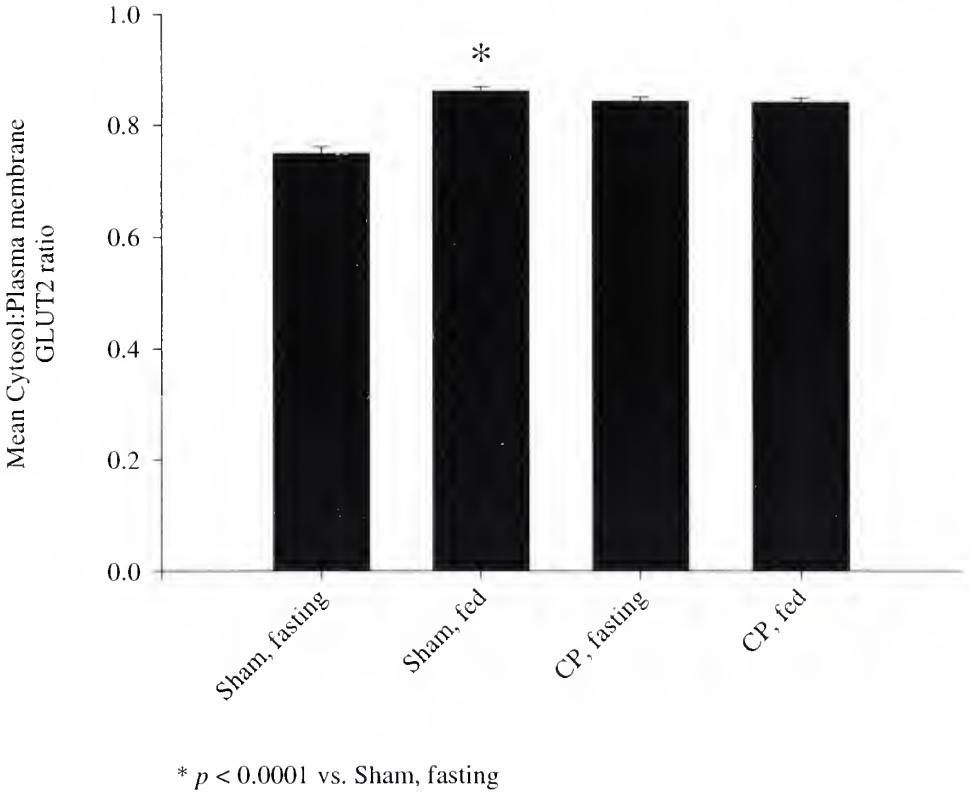


Figure 22. Cytosol (CYT)-to-plasma membrane (PM) ratio of GLUT2 immunofluorescence in sham and chronic pancreatitic (CP) rat livers, as determined by digital confocal laser scanning microscopy. The CYT:PM ratio of GLUT2 increased from 0.75 ± 0.01 (mean ± SEM) in fasting sham rat livers to 0.86 ± 0.01 in fed sham rat livers ($p < 0.0001$). There was no change in the CYT:PM ratio of GLUT2 in CP livers following duodenal feeding.

DISCUSSION

The association between chronic pancreatitis and impaired glucose tolerance has been known for over two centuries, since Cawley's report in 1788 (1). Approximately 70% of patients with CP manifest impaired oral glucose tolerance, and up to 35% of pancreatitic patients develop frank diabetes mellitus requiring insulin therapy (13-15). Although diabetic ketoacidosis and the classic long term microangiopathic complications of diabetes are rare in diabetes secondary to CP, these patients are very prone to iatrogenic hypoglycemia with resultant sudden, wide fluctuations in blood glucose levels—referred to as "brittle" diabetes (3-8). The difficulty in maintaining glycemic control presents one of the greatest challenges in managing the patient with chronic pancreatitis. By better understanding the hepatic glucoregulatory mechanism and dysfunction in pancreatogenic diabetes, methods of improving the clinical outcome of patients with chronic pancreatitis or following pancreatic resection will be developed.

Utilizing euglycemic glucose-clamp techniques, prior studies in a dog model of CP and in chronic pancreatitic patients have shown that inappropriately high rates of hepatic glucose production are maintained in the face of exogenously elevated insulin levels (29,32-35). *Ex vivo* isolated liver perfusion studies have also demonstrated a loss of insulin-mediated suppression of glucagon-stimulated hepatic glucose production in CP livers (30,31). Although it is well-established that this reduced sensitivity to insulin is specific for the liver and contributes to the abnormal glucose metabolism seen in CP (29-31), the mechanism of hepatic resistance to insulin remains largely unknown.

As it has been demonstrated that states of insulin resistance, including obesity, Type II diabetes, pregnancy, and glucocorticoid excess, are associated with diminished insulin binding due to either decreased IR availability or reduced IR affinity (61-76), a reduced expression of insulin receptors on the hepatocyte membrane may play a role in the hepatic insulin resistance in CP. Indeed, competitive insulin binding studies have indicated that CP hepatocytes exhibit diminished insulin binding due to a reduction in the number of plasma membrane-bound insulin receptors (77).

Abnormalities in GLUT2 availability on the cell membrane may also have a role in hepatic insulin resistance in CP. Studies have demonstrated that failure of insulin-induced suppression of hepatic glucose production in CP is associated with absence of insulin-mediated reduction in hepatocyte plasma membrane-bound GLUT2 (31). As the cell membrane availability of proteins is regulated, in part, by the intracellular trafficking and recycling of newly synthesized and internalized proteins, the abnormalities in insulin receptor and GLUT2 availability in chronic pancreatitis may be the result of altered intracellular vesicle trafficking and may play a role in the hepatic insulin resistance and glucose intolerance associated with chronic pancreatitis.

Modes of Endocytosis

Endocytosis is the process of internalization of material from the extracellular environment by invagination of the cellular membrane and can be divided into two types: (a) phagocytosis, which involves internalization of material that is visible by light microscopy, and (b) pinocytosis, which describes the internalization of insoluble

particles, low molecular weight solutes, and extracellular fluid (131,132). Pinocytosis can be further classified as fluid-phase pinocytosis—or "fluid-phase endocytosis" (FPE), defined as uptake of materials that are within the extracellular fluid, and adsorptive pinocytosis, defined as uptake of materials that are bound to the extracellular surface of the plasma membrane (131). Finally, adsorptive pinocytosis can be subdivided into two processes depending upon whether the material is bound in a non-specific manner or bound via specific receptors—the latter is referred to as "receptor-mediated endocytosis" (RME; 131-133).

In the first portion of the current study, we utilized a marker of FPE, fluorescein-isothiocyanate labeled 70-kDa dextran, in order to assess whether endocytic vesicle trafficking is impaired in rats with chronic pancreatitis. Studies have demonstrated that in hepatocytes, the vast majority of cellular fluid-phase uptake is due to FPE and that RME contributes only a few percent of the total uptake of fluid (134,135). Furthermore, the pathway of FPE contributes negligibly to the uptake of ligands that bind to specific cell surface receptors. These unique characteristics allow investigation of endocytosis in hepatocytes using markers that are taken up exclusively in the fluid phase, independent of the RME pathway.

FITC-D has been used extensively as a marker of FPE in hepatocytes (122,124), kidney cells (123,125), fibroblasts (121), and macrophages (126). Several properties define FITC-D as a marker taken up in the fluid-phase: (a) uptake is directly proportional to the concentration in the surrounding medium, (b) uptake proceeds linearly with time and does not alter ongoing FPE, (c) the marker is not adsorbed to the cell surface prior to

internalization, and (d) uptake is temperature-dependent, with lower temperatures reducing internalization (136).

Our results indicate that sham rat hepatocytes *in vivo* exhibit a reduction in FPE in response to feeding, as measured by FITC-D uptake. As shown in our model recapitulating feeding in sham rats, this change in endocytosis occurs within 45 minutes following duodenal feeding of a liquid meal, while in fasting sham livers, FPE increases linearly during the experimental period. The finding that feeding induces a reduction in FPE in normal hepatocytes is a novel observation, and the mechanism and significance of this feeding-induced response requires further investigation.

Implications of Reduced Fluid-Phase Endocytosis

Evidence has suggested that inter-relationships may exist between different endocytic processes. In 1977, Bowers demonstrated in *Acanthamoeba* that concomitant phagocytosis caused a reduction in the rate of [^{3}H]-inulin internalization by FPE (137). Furthermore, the study indicated that the total volume of material internalized by phagocytosis and pinocytosis remained constant over time. These results support the notion that in some cell types the rate of endocytosis may be determined by the total cellular volume occupied by material that has been internalized. Damke et al. have shown that inhibition of clathrin-dependent RME induces clathrin-independent FPE in HeLa cells expressing a temperature-sensitive mutant form of dynamin, a GTPase required for endocytosis via the clathrin-coated pit pathway (138). Within five minutes of exposure to non-permissive temperatures, RME is inhibited, and FPE of horseradish

peroxidase continues at a reduced level. Within thirty minutes of continued exposure, the rate of FPE recovers to levels of wild-type HeLa cells, while RME remains inhibited. This study supports the prevailing view that clathrin-dependent RME and clathrin-independent FPE occur concomitantly and that inhibition of the clathrin-dependent pathway induces upregulation of the clathrin-independent pathway (138,139).

Other reports have shown that a coupling of the processes of endocytosis and exocytosis exists in cells. In 1973, Orci et al. provided evidence that glucose-stimulated exocytosis of insulin by the pancreatic β-cell is accompanied by an increase in the internalization of horseradish peroxidase by FPE (140). A recent study has suggested that the rate of membrane retrieval—i.e., endocytosis of the plasma membrane—in β-cells is rate-limiting for the exocytosis of insulin (141). Kalina et al. demonstrated that exocytosis of zymogen granules in parotid acinar cells is associated with extensive internalization of ferritin by adsorptive endocytosis (142). Similarly, Wahlin et al. found that discharge of secretory granules by gallbladder epithelial cells from mice is accompanied by increased uptake of thorium dioxide by pinocytosis (143). Recently, cell capacitance studies in pituitary melanotrophs (144), neuronal synaptic terminals (145), and adrenal chromaffin cells (146) have shown that stimulated exocytosis is followed by a rapid burst of endocytosis to maintain cell surface area constant and to retrieve membrane for vesicle recycling. Thus, although the physiologic significance of coupled endocytosis-exocytosis requires further study, it is likely that coupling may represent a mechanism by which plasma membrane is conserved by the cell, and membrane constituents are recycled.

We postulate that an analogous inter-relationship exists between different modes of endocytosis in the rat hepatocyte. It is well-known that feeding induces an increase in blood insulin levels by glucose-stimulated secretion from the pancreatic β-cells. Following the binding of insulin to its receptor on the plasma membrane, internalization of the insulin-IR complex occurs by RME (147-149). Our data suggest that FPE in hepatocytes, a constitutive process, is interrupted when RME of the insulin-IR complex is initiated. Thus, the reduction in FPE in sham rat liver may be a compensatory mechanism whereby the cell is able to limit internalization of plasma membrane, minimize energy expenditure for "housekeeping" processes, or maintain constant cellular volume—or surface-to-volume ratio—during the increase in a receptor-mediated event. The "shift" in endocytic activity from FPE to RME may also indicate that vesicle trafficking proteins are differentially oriented from FPE-associated vesicles upon signaling from specific ligand-bound receptors. It remains to be determined whether a coupling exists between FPE and RME in the rat hepatocyte and what the mechanism of such a coupling may be at the cellular level.

Absence of Reduced Fluid-Phase Endocytosis in Chronic Pancreatitis

Although the possible explanations for the feeding-induced reduction in FPE in sham rat hepatocytes remain teleological, our study demonstrates that in response to feeding, FITC-D uptake in hepatocytes from rats with CP is not interrupted. In the FITC-Dextran Time-Course Study, CP hepatocytes continue to accumulate FITC-D over time following administration of a duodenal meal, suggesting that FPE remains unaltered in

response to feeding. The impairment in feeding-induced changes in FPE in CP hepatocytes may be a manifestation of a generalized cellular defect in intracellular vesicle trafficking.

Abnormalities in intracellular vesicle trafficking have been implicated in the altered plasma membrane availability of various cell surface receptors in hepatocytes. In 1987, Casey et al. suggested that impaired receptor recycling results in a reduced number of hepatocyte plasma membrane-bound asialoglycoprotein receptors in ethanol-fed rats (102). Furthermore, Carpentier et al. demonstrated that exposure of a lymphocyte cell line to monensin, an inhibitor of receptor recycling, inhibited the insertion of recycled IR into the plasma membrane (104). Our results indicate that feeding-induced changes in FPE in hepatocytes of rats with chronic pancreatitis are impaired. Whether this represents a reduced level or even a failure of RME in CP with a consequent unchanged level of FPE, as suggested by a coupling model of RME and FPE, is open to investigation.

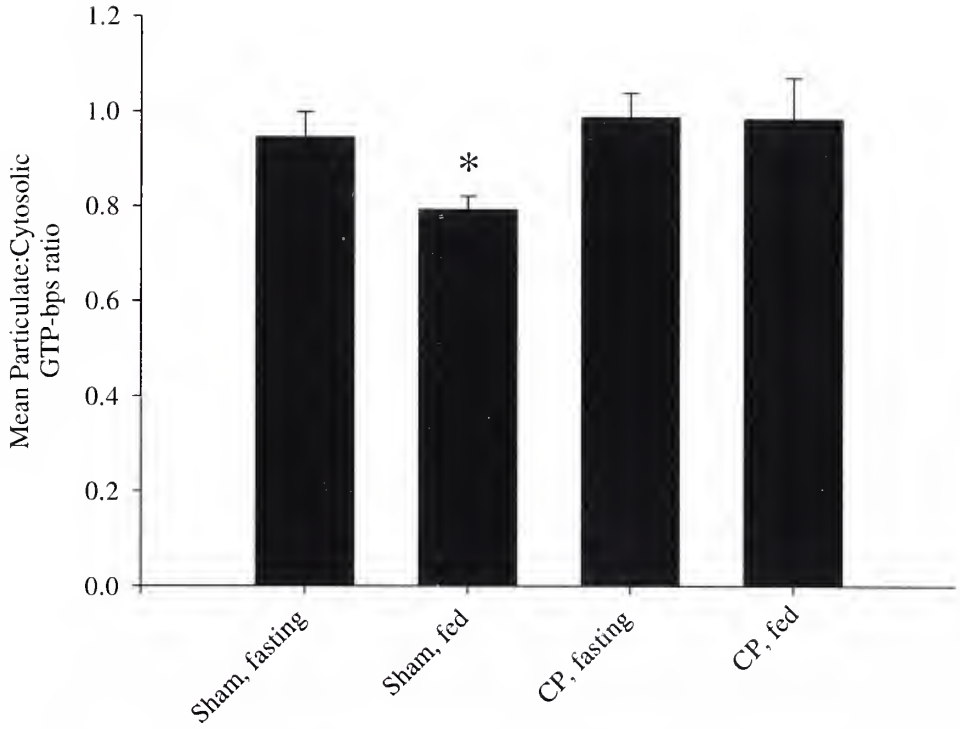
It is evident from our data that a large degree of intra-group variability exists between the CP rat livers with regard to their FITC-D uptake over time. Because chronic pancreatitis as a disease may affect individuals with varying severity, it is not unexpected that our results exhibit impressive inter- and intra-animal variability with respect to their FPE. Despite a large degree of variance in the data obtained in the CP animals, our results are nonetheless consistent with an alteration in hepatocyte endocytosis in this model of disease.

Correlation with Low Molecular Weight GTP-Binding Proteins Study

The cellular mechanism responsible for the impairment in feeding-mediated FPE changes in CP is not yet determined. However, a recent investigation of the subcellular distribution of low molecular weight GTP-binding proteins in livers from CP rats suggests that altered expression or distribution of vesicle trafficking proteins may be responsible for the changes observed.

The *ras*-superfamily of monomeric, low molecular weight GTP-bps consists of at least 30 members, some of which have been implicated in the regulation of intracellular vesicle trafficking (105-110). A recent study by Juvet et al. demonstrated that the rate of internalization of the galactose receptor by rat liver cells correlates with the level of expression of one such GTP-binding protein, rab5 (110). In 1992, Bucci et al. found that the reduced rate of FPE of horseradish peroxidase in kidney cells was due to overexpression of a mutant form of rab5 (107). We therefore investigated whether alterations in GTP-bps may play a role in the disordered FPE observed in CP rat livers.

In a previous study in our laboratory, we utilized [α-^{32}P]-GTP binding assays to determine whether the distribution of low molecular weight GTP-bps is altered in fractionated livers from rats with CP. We found that livers from sham rats in the fed state exhibited a reduction in the particulate-to-cytosolic ratio of a subgroup of low molecular weight GTP-bps, as compared to sham rats in the fasting state (unpublished data; Figure 23). Moreover, the particulate-to-cytosolic ratio of the low molecular weight GTP-bps was unchanged in CP livers following feeding, as compared to CP livers from fasting rats. These findings correlate well with the results of our FITC-Dextran Infusion Study



* $p < 0.05$ vs. Sham, fasting

Figure 23. Particulate-to-cytosolic ratio of GTP-bps ("peak 1" subgroup) in sham and CP rat livers, as determined by [α-^{32}P]-GTP binding assays and scanning densitometry. Liver homogenates were centrifuged at 200,000 x g for 60 minutes to obtain cytosolic and particulate fractions. Following SDS-PAGE and electrotransfer to nitrocellulose, membrane was incubated with [α-^{32}P]-GTP for 48-72 hours and exposed to Kodak X-OMAT AR film. GTP-bps were quantified by scanning densitometry and area quantitation analysis. Six livers were studied from fasting sham and fed sham groups, and eight were studied from fasting CP and fed CP groups.

(compare Figure 10), in which we found that FITC-D uptake in fasting sham rat hepatocytes decreased in livers from sham rats that were fed, while FITC-D internalization remained unchanged in CP rats. This correlation supports the notion that

low molecular GTP-bps may be critical factors in the regulation of FPE and intracellular vesicle trafficking in rat liver, and that the loss of regulation of vesicle trafficking due to alterations in the distribution of GTP-bps may play a role in the disordered FPE in chronic pancreatitis.

Implications of Alterations in GLUT2 Internalization

As discussed above, transmembrane glucose transport in the hepatocyte is catalyzed by the facilitated-diffusion transporter protein, GLUT2. GLUT2 is located in the sinusoidal membrane of hepatocytes, and over 90% of the protein is present in the cell membrane in the basal state (80,81,86). Early studies failed to show any further increase in hepatocyte plasma membrane GLUT2 availability, and it had previously been thought that insulin has no effect upon GLUT2 distribution in the liver. However, in 1994, Andersen et al. demonstrated in *ex vivo* isolated liver perfusion experiments that insulin mediates a reduction in hepatocyte plasma membrane-bound GLUT2 and that this effect correlates directly with the insulin-induced suppression of hepatic glucose production (31).

In the second portion of the current study, we utilized subcellular fractionation and confocal imaging techniques to determine whether the insulin-mediated reduction in hepatocyte plasma membrane-bound GLUT2 is the result of active internalization of GLUT2 into the endosomal pool and whether this process is disturbed in CP. Our results indicate that a 15% to 53% shift in hepatocyte GLUT2 occurs from the plasma membrane to the endosomal pool in sham rats following feeding. In the fractionation study, we

found a 60% increase in the GLUT2 content of the E-enriched fraction of sham rat liver following the duodenal meal. However, a concomitant statistically significant reduction in the GLUT2 content of the PM-enriched fractions was not observed. One explanation for this lack of an observed change in PM-associated GLUT2 is that a relatively modest shift in GLUT2 from the plasma membrane pool may not have been evident within the context of a very large amount of plasma membrane GLUT2 in the basal state. By increasing the sample size—and thus, the power of our study, it is likely that we would be able to detect a very small reduction in the quantity of plasma membrane GLUT2 following feeding in sham rats. In addition, due to the large quantity of GLUT2 in the PM-enriched fractions, it is possible that our immunoblot exposures were too saturated to detect a statistically significant reduction in PM-associated GLUT2.

In the confocal imaging study, we observed a 13% reduction in plasma membrane GLUT2 following feeding in sham rats, but cytosolic GLUT2 immunofluorescence remained unchanged. A 16 x 16-pixel area of cytosol was used to quantify internalized GLUT2 in the hepatocytes—i.e., GLUT2 that had shifted from the plasma membrane to the endosomal pool following feeding. The lack of a concomitant statistically significant increase in the cytosolic GLUT2 content was likely due to the fact that the "cytosolic" GLUT2 detected by confocal immunofluorescence is not a true measure of endosomal GLUT2, which we were able to quantify in the fractionation study. The confocal microscopy study was unable to differentiate internalized GLUT2 from GLUT2 that was being newly produced and was localized to the endoplasmic reticulum and to the Golgi complex. An additional confounding factor was that the "cytosolic" compartment included non-GLUT2 background artifact due to some non-specific binding by the anti-

GLUT2 antibody. Thus, it is conceivable that if the quantity of non-endosomal GLUT2 in the cytosolic compartment was large, the increase in endosomal GLUT2 following feeding may not have been detectable by this technique. Nevertheless, the finding that plasma membrane GLUT2 is reduced following feeding in sham rats is consistent with the results of our fractionation study and further supports our hypothesis that feeding induces a shift in GLUT2 from the plasma membrane to the endosomal pool.

Our finding that feeding mediates GLUT2 internalization in sham rat hepatocytes is a novel observation and corroborates our prior results of an insulin-induced reduction in hepatocyte plasma membrane-bound GLUT2 (31). However, the mechanism of and the metabolic consequences of GLUT2 internalization remain to be elucidated. As described above, it is well-known that internalization of the insulin-IR complex occurs following binding of the hormone to its cell surface receptor (55-59). Thus, we postulate that the feeding-induced internalization of GLUT2 in sham rat livers is regulated by the receptor-mediated endocytosis of the insulin-IR complex. Whether the insulin-IR complex and GLUT2 are co-localized to the same endocytic vesicles or GLUT2 is internalized as a downstream event in the insulin-IR signaling cascade is yet to be determined.

Several studies have elucidated the role of insulin in the regulation of facilitative glucose transporter proteins (GLUT4) in adipose and skeletal muscle tissue. In adipocytes and skeletal muscle cells, GLUT4 is predominantly located in cytoplasmic vesicles (93,95-97,150,151). Insulin causes translocation of the vesicles to the plasma membrane—with involvement of another GTP-binding protein, rab4 (152,153), an increase in the plasma membrane-bound quantity of GLUT4, and the resultant

enhancement of the rate of glucose internalization by the cell (95,98-101). In a recent study, Dombrowski et al. demonstrated that impaired GLUT4 translocation in skeletal muscle in streptozotocin-induced diabetes results in altered glucose metabolism (154). The dependence of the rate of glucose transport upon the availability of membrane-bound GLUT4 is a function of its intermediate K_m (2-10 m*M*). Thus, at physiologic glucose concentrations, GLUT4 may be highly saturated, thereby causing glucose metabolism to be limited by the rate of transmembrane transport.

The potential role of GLUT2 internalization in the insulin-induced suppression of hepatic glucose production is unknown. It is possible that the feeding-induced, or insulin-mediated, GLUT2 internalization in hepatocytes may function to reduce glucose release from the liver in the post-prandial state when further hepatic glucose production would otherwise interfere with the maintenance of appropriate glucose homeostasis. Although the high K_m of GLUT2 suggests that a large change in the plasma membrane-bound quantity of the protein is necessary to effect a change in the rate of glucose transport, this notion is largely speculative and is further complicated by the fact that glucose transport in the hepatocyte is bi-directional.

Absence of GLUT2 Internalization in Chronic Pancreatitis

The results of our subcellular fractionation and confocal microscopy studies indicate that the feeding-induced GLUT2 internalization observed in sham rats is absent in rats with oleic acid-induced chronic pancreatitis. The mechanism for this impairment in GLUT2 internalization is not yet known. However, our findings of impaired feeding-

induced suppression of FPE in CP suggest that a generalized disturbance in intracellular vesicle trafficking in CP may be responsible for these results. A diminished number of plasma membrane-bound insulin receptors in the basal state (77), the loss of the insulin-induced change in GLUT2 availability (31)—likely a manifestation of failed GLUT2 internalization—and the absence of the feeding-induced reduction in fluid-phase endocytosis may all be related to a defect in the trafficking of intracellular vesicles containing IR and/or GLUT2.

The altered expression of glucose transporters has been studied most extensively in the pancreatic β-cell. In streptozotocin-induced diabetic rats whose β-cells exhibit impaired glucose-stimulated insulin release, Thorens et al. found that the severity of hyperglycemia correlated with the extent of the reduction in the expression of GLUT2 on the β-cell surface (88). In 1992, Ogawa et al. demonstrated in dexamethasone-induced diabetic rats that absent insulin response to glucose by the pancreatic islets is associated with diminished GLUT2 expression and reduced glucose transport in the β-cells (89). Furthermore, it has recently been demonstrated in a mouse model of streptozotocin-induced diabetes that a significant reduction in GLUT2 protein and mRNA expression in pancreatic islets develops prior to the manifestation of diabetes (91). Thus, the diminished expression of GLUT2 on the plasma membrane results in a decreased rate of glucose transport into the β-cell, further supporting the notion that GLUT2 is a critical factor in the loss of glucose-induced insulin secretion in some diabetic states (92).

Whether the absence of feeding-induced GLUT2 internalization in CP livers contributes to the persistent hepatic glucose production and abnormal glucose metabolism

of chronic pancreatitis requires further study. We have previously demonstrated that hepatocyte membrane-bound GLUT2 availability is related to the prevailing level of glucose production by the liver (31). The current studies suggest that failed GLUT2 internalization may be responsible, in part, for persistent hepatic glucose release and resultant hyperglycemia in the post-prandial state in chronic pancreatitis.

Summary

Chronic pancreatitis is associated with impaired glucose tolerance, reduced hepatic sensitivity to insulin, and a loss of insulin receptor availability on hepatocyte plasma membranes. In normal and sham rats, the insulin-induced suppression of hepatic glucose production is associated with a decrease in the quantity of hepatocyte plasma membrane-bound GLUT2. This insulin-mediated response is absent in chronic pancreatitis.

Within forty-five minutes following feeding, normal rat hepatocytes *in vivo* exhibit a reduction in fluid-phase endocytosis, as measured by uptake of FITC-Dextran. The physiological significance of this reduction is unknown but may represent an interruption of fluid-phase endocytosis when receptor-mediated endocytosis of the insulin-insulin receptor complex is initiated. The feeding-induced reduction in fluid-phase endocytosis observed in sham rat hepatocytes is lost in hepatocytes from rats with chronic pancreatitis. Whether this is a manifestation of a generalized defect in intracellular vesicle trafficking in chronic pancreatitis requires further investigation. Such a defect in vesicle trafficking could be a factor in the altered plasma membrane

availability of hepatic insulin receptors and facilitated-diffusion glucose transporters (GLUT2) in chronic pancreatitis.

The loss of feeding-induced changes in fluid-phase endocytosis in hepatocytes from chronic pancreatitic rats correlates with changes in the distribution of low molecular weight GTP-binding proteins which are involved in intracellular vesicle trafficking. Thus, altered regulation of vesicle trafficking may play a role in the disordered regulation of endocytosis in chronic pancreatitis, and in the hepatic insulin resistance and resultant glucose intolerance associated with the disease.

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